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Production, purification, and characterization of a glucoamylase from Thermoanaerobacterium thermosaccharolyticum

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Production, purification, and characterization of a glucoamylase from *Thermoanaerobacterium thermosaccharolyticum*

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INTRODUCTION

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Glucoamylase and its diverse forms

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3, GA) is an exo-acting enzyme that catalyzes the release of β-D-glucose from the non-reducing ends of starch or related oligosaccharides and polysaccharides. It is also known as amyloglucosidase and earlier as gluc-amylase and λ-amylase. GA is an industrially important enzyme in the starch bioprocessing and brewing industries.

GAs can be produced by various microbial organisms, such as fungi, yeast, and bacteria. They differ primarily in molecular weight, amino acid sequence, and capacity to bind to starch granules (Saha and Zeikus, 1989a). The industrial focus has been on GAs from fungi belonging to the species *Aspergillus niger* (identical to *Aspergillus awamori* GA) and *Rhizopus oryzae* (Saha and Zeikus, 1989a), but GAs from yeasts of the genus *Saccharomycopsis* also offer potential.

Multiple forms of GA, called isoenzymes, have been found in fungi. *A. niger* can produce GA in six forms, of which the two major forms are GAI and GAII (Ono et al., 1988). GAI contains 616 amino acid residues, while GAII, which is a digestion product from GAI proteolysis, contains a mixture of 512 and 514 amino acid residues (Svensson et al., 1982, 1986; Nunberg et al., 1984). The cloned GA genes of *A. awamori*, *A. niger*, and *A. awamori* var. *kawachi* encode for full-length GAs containing 615 or 616 amino acid residues and a signal peptide at the *N*-terminus, which is removed during enzyme secretion (Svensson et al., 1983; Nunberg et al., 1984). GAI has two domains: a catalytic domain (CD) containing residues 1–467, which includes an *O*-glycosylated region from residues 441–467; and a starch-binding domain (SBD) containing residues 509–606, which can bind insoluble starch and enhances the hydrolysis of insoluble starch by the catalytic domain. They are connected by a linker

region containing residues 468–508, which has many threonine and serine residues that are almost all *O*-linked to mannose (Coutinho and Reilly, 1994b) (Figure 1). The molecular weights of the polypeptide moiety (derived from the amino acid sequence) and the total GAI molecule are 65,424 and 82,000, respectively (Svensson et al., 1983).

Three GA forms resulting from differential proteolytic cleavage have been observed in *R. oryzae* (Tanaka et al., 1986a). Two different GAs with distinct catalytic properties are produced by *Hormoconis resinae* (Fagerström et al., 1990). In addition, *Saccharomyces diastaticus* and *Schizosaccharomyces pombe* produce varied forms of yeast extracellular GAs (Yamashita et al., 1985).

There are several possible reasons to explain the multiplicity of GA forms. Diverse forms can be produced by stepwise degradation of native GA with proteases or glycosidases in the course of mold cultivation and enzyme preparation (Hayashida et al., 1976, 1978). Medium composition and culture conditions of the microorganisms affect the diverse forms of GA (Lineback et al., 1966; Barton et al., 1969). Purification procedures also influence the number of discrete forms of GA from *A. niger* (Fleming and Stone, 1965). Post-translational modification or phosphorylation (Ferro-Luzzi Ames and Nikaido, 1981; Celis, 1984) can also lead to the occurrence of multiple forms of GA.

Physicochemical characteristics of GAs

Molecular weights of fungal GAs vary from 48 to 112 kDa (Manjunath et al., 1983; Saha and Zeikus, 1989a). The same range holds true for yeast GA except some forms can range up to 250 kDa, such as GA produced in *S. diastaticus* GA (Yamashita et al., 1985). Molecular weights associated with the mature sequenced GAs (not including carbohydrate moieties) are between 55 kDa for *Saccharomycopsis fibuligera* GA and 104 kDa for *Schwanniomyces occidentalis* GA (Itoh et al., 1987; Dohmen et al., 1990).

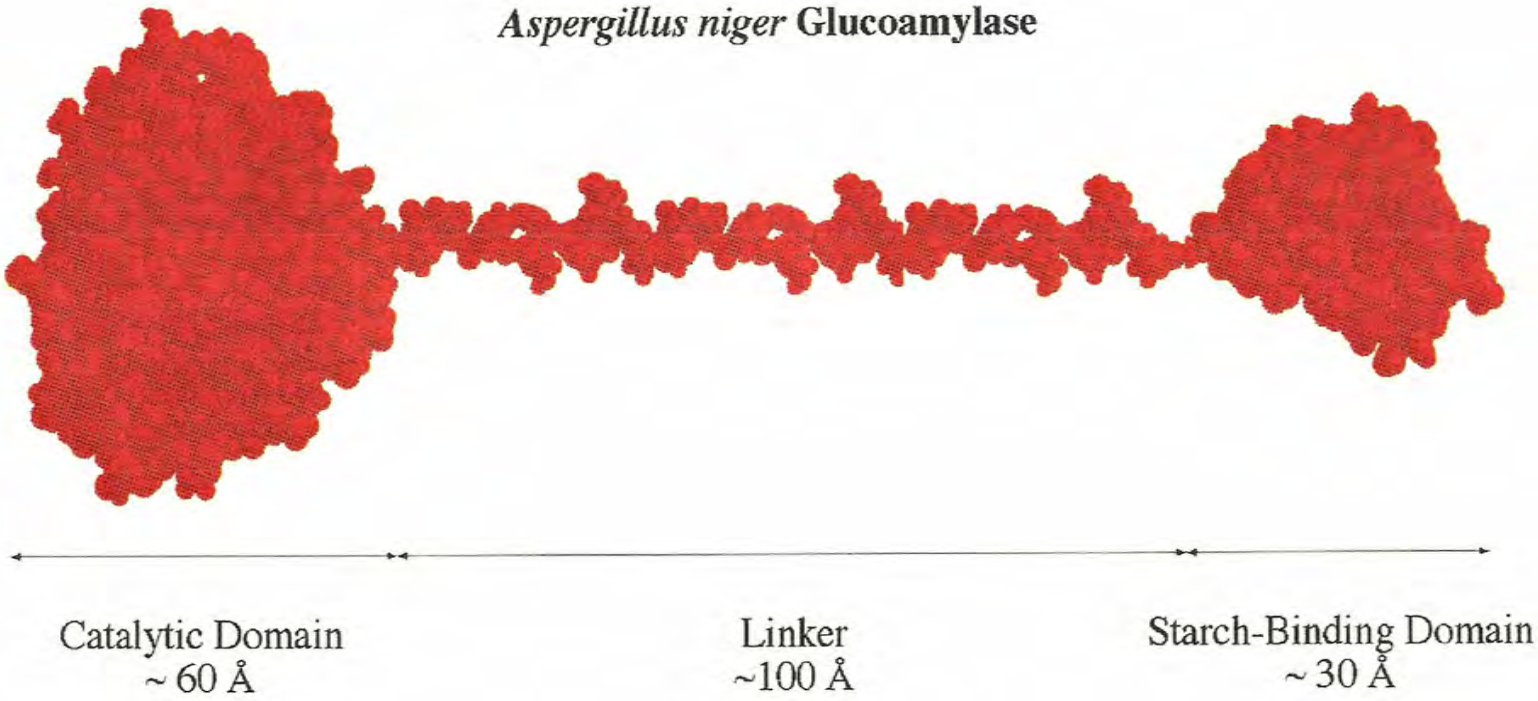

Figure 1. Schematic structure of *Aspergillus niger* GA. The catalytic domain used is GA from *Aspergillus awamori* var. X100 (1gly), the linker was built from repetitions of the *O*-glycosylated belt around 1gly, and the starch-binding domain is domain E of cyclodextrin glucosyltransferase from *Bacillus circulans* no. 8 (1cgt) (Coutinho, 1996).

GAs operate optimally at acidic pH and relatively high temperatures (Manjunath et al., 1983; Saha and Zeikus, 1989a). The optimal pH for GAs is generally between 4 and 5, but examples of 3.5 and 6.5 have also been reported (Manjunath et al., 1983). The optimal pH for GA stability is situated between 3 and 8, with isoelectric points (pIs) between 3.4 and 7 (Saha and Zeikus, 1989a). Approximate pIs calculated from mature GA sequences vary from 4.0 in *A. niger* GA to 6.9 in *R. oryzae* GA. GAs show a temperature optima of 40~60°C. At higher temperatures, the enzyme is rapidly and irreversibly inactivated (Munch and Tritsch, 1990; Chen et al., 1994).

Fungal and yeast GAs are glycoproteins containing about 3–30% carbohydrate, which is generally composed of mannose, glucose, galactose, and glucosamine (Manjunath et al., 1983; Saha and Zeikus, 1989a). *N*- and *O*-glycosylation can be found in *Aspergillus* and *Rhizopus* GAs (Pazur et al., 1990). Different glycosylation levels may cause the variety of GA forms found in some species. A hyperglycosylated GA produced by a strain of *A. awamori* var. *kawachi* enhances the binding ability to raw starch (Fukuda et al., 1992). Other modifications of the carbohydrate component, such as occurred in *A. awamori* var. *X100* GA, leads to variations in temperature and pH stability (Neustroev et al., 1993a,b).

Since GAs from bacteria are not glycosylated, variations in glycosylation as an explanation for the existence of multiple forms of bacterial GAs can be excluded.

Industrial applications of GAs

GA has been regarded as an industrially important enzyme, especially in the starch and brewing industries. The most important application of GA is in the conversion of starch dextrins to high-glucose syrups. The process includes liquefaction of starch either by acidic hydrolysis or α-amylase at high temperature, then saccharification by GA alone or in association with a debranching enzyme such as pullulanase (Saha and Zeikus, 1989a). Glucose isomerase is then used to convert glucose to fructose. Enzymatic hydrolysis is preferred to

acid hydrolysis in starch processing because fewer undesirable byproducts (Reilly, 1985) and off-flavors are produced (Teague and Brumm, 1992).

GA is also used in ethanol production for fuel, chemicals, or spirits (Nikolov and Reilly, 1991; Sakaguchi et al., 1992). Industrial yeasts are usually unable to completely ferment starch, and therefore pretreatment of starchy materials by α-amylase liquefaction and GA saccharification is necessary (Saha and Zeikus, 1989a). In Japan, fungal *Aspergillus* and *Rhizopus* species are used separately or together for ethanol production (Sakaguchi et al., 1992).

To improve ethanol production by fermentation, newly characterized GA genes have been introduced into yeast and fungi. For example, the GA genes of *S. diastaticus* and *S. occidentalis* were cloned into *Saccharomyces cerevisae* (Errat and Nasim, 1987; Dohmen et al., 1990), the *A. shirousami* GA gene was cloned into *A. oryzae* (Shibuya et al., 1990), and the GamP gene of *H. resinae* was cloned into *Trichoderma reesei* (Joutsjoki et al., 1993). The main purpose of these transfers was to introduce a better and secretable GA.

GA is extensively used in the production of light beer (Sills et al., 1983b). The main source of calories in beer is dextrins (70 to 75% branched), which cannot be metabolized by traditional beer strains. Dextrins can be easily hydrolyzed after adding the fungal GAs, and a beer with lower caloric content can be obtained. The GA produced by the yeast *S. occidentalis* is thermolabile and therefore the pasteurization step (8 min at 60°C) to avoid sweetening the beer due to hydrolysis of residual dextrins during storage (Sills et al., 1983a,b) can be eliminated. An attempt to construct hybrid beer-producing *Saccharomyces* strains capable of secreting GA has also been reported (Sills et al., 1983b; Hansen et al., 1990).

Limitations of fungal GAs for industrial processes

Fungal GAs, although highly effective in hydrolyzing starch, can only catalyze the saccharification reaction within a relative narrow temperature range because the catalytically act-

ive conformation changes at elevated temperatures. This limited thermostability affects their use in industrial processes where prolonged incubation at high temperatures is required. For instance, in the conventional high-glucose syrup production, the starch slurry (30–40% DS) is first liquefied at high temperature with thermostable α-amylase (105°C for 5 min, then 95°C for 2 h, pH 6.0–6.5) and then hydrolyzed to glucose with fungal GA at low temperature (60°C, pH 4.0–4.5 for 48–72 h) (Saha and Zeikus, 1989b). Separate tanks for liquefaction and saccharification with a cooling system between them are required due to the difference of operational temperatures, and this contributes to the overall process time and cost. Development of a thermostable GA would greatly improve the process by combining two reactions in one tank, allowing higher temperature to increase the reaction rate. Other advantages of adopting a higher operational temperature include lower probability of microbial contamination and reduced fluid viscosity.

GA thermostability and strategies to enhance it

For an enzyme to be active, proper folding is required to ensure structural and functional integrity of the active domain, where substrates can be bound and interact with the catalytic residues. At elevated temperatures, extensive intramolecular motion and covalent chemical reactions take place and subsequently lead to disruption of the active conformation and then loss of activity (Ahern and Klibanov, 1988).

Many strategies are available to stabilize an enzyme. Traditional methods include chemical modification and immobilization. Chemical modification has resulted in remarkable stabilization of some enzymes, including trypsin and α-chymotrypsin (Mozhaev et al., 1988); however, its application to GA generally yields activity loss or little increased stability (Sinitsyn et al., 1978; Munch and Tritsch, 1990). Immobilization of GA would allow continuous saccharification and improved enzyme thermostability compared to soluble forms; however, it has not been implemented industrially due to the problems of activity loss, insufficient

stabilization, or decreased glucose yields in maltodextrin saccharification (Lee et al., 1976; Przybyt and Sugier, 1988). There is no GA with increased practical industrial stability so far that resulted from conventional enzyme stabilization methods, and new technologies need be investigated for this to occur.

Protein engineering is a new technology that provides researchers with more tools for enzyme modification. Both random and site-directed mutagenesis have been used to introduce stabilizing amino acid substitutions into enzymes. These two powerful tools have led to the isolation of several mutants with increased thermostability. Two general reviews of improving performance of GA by mutagenesis have appeared recently (Ford, 1999; Reilly, 1999).

GAs from thermophilic bacteria

A more direct approach than mutagenesis of less thermostable GAs is to isolate thermophilic microorganisms that may produce thermostable GAs. Thermophilic microorganisms are characterized by their ability to grow at temperatures higher than 60°C, with optimal temperatures above 55°C. Extreme thermophiles have temperature optima above 65°C, and hyperthermophiles can grow optimally at temperatures above 80°C. According to the above definition, true thermophiles have been described only in the prokaryotic organisms, including aerobic and anaerobic bacteria as well as archaea.

Over the past fifteen years, a variety of anaerobic and aerobic bacteria have been investigated for their ability to produce thermoactive starch-hydrolyzing enzymes. Most of these microbes secrete thermostable amylases and pullulanases (Antranikian et al., 1987; Antranikian, 1989, 1990). Enzymes subjected to detailed biochemical studies have been from anaerobic bacteria belonging to the genera *Clostridium*, *Thermoanaerobium,* and *Thermoanaerobacterium* (Plant et al., 1987; Saha et al., 1988; Spreinat and Antranikian, 1990; Specka et al., 1991; Ganghofner et al., 1998). Unlike amylases and pullulanases, GA activities expressed in prokaryotes are very low, while their activities in fungi as well as some yeasts

are much higher. However, GA activity has been detected in a few aerobic bacteria such as *Bacillus stearothermophilus*, *Flavobacterium sp.*, and *Halobacterium sodomense* (Bender, 1981; Oren, 1983), and also in the anaerobes *Clostridium acetobutylicum, Clostridium thermohydrosulfuricum*, *Clostridium* (now *Thermoanaerobacterium* (Collins et al., 1994)) *thermosaccharolyticum* DSM 571 and 572, and other *Clostridium* species (Hyun and Zeikus, 1985; Chojecki and Blaschek, 1986; Fagerström, 1991; Ohnishi et al., 1991, 1992; Specka et al., 1991; Ganghofner et al., 1998).

GAs from thermophilic anaerobic bacteria like *Clostridium sp. G0005* and *T. thermosaccharolyticum* DSM 572 can be produced both in the cells and in the medium (Ohnishi et al., 1991; Specka et al., 1991). A cell-bound GA (cGA) from *Clostridium sp. G0005* has been extensively characterized. The optimal pH and temperature for this cGA is 4.5 and 65°C. It is stable at 60°C but can be inactivated above 65°C (Ohnishi et al., 1991). The gene coding for cGA has been sequenced and it was the first bacterial GA to be cloned (Ohnishi et al., 1992). *Clostridium sp. G0005* cGA differs from other GAs in its high activity toward 1,6-glucosidic bonds, with its K_m and k_{cat} for isomaltose being 77-fold smaller and 22-fold larger, respectively, than those of the *A. niger* enzyme (Ohnishi et al., 1992). Comparison of the deduced amino acid sequence of cGA with several fungal GAs showed low similarity over the entire primary structure (Ohnishi et al., 1992), but four of the five highly conserved regions that are found in fungal GAs (Tanaka et al., 1986b; Itoh et al., 1987) also occur in *Clostridium sp. G0005* cGA (Coutinho and Reilly, 1997). However, cGA is less homologous in these four regions than the other enzymes.

GA from *T. thermosaccharolyticum* DSM 572 has also been studied in detail (Specka et al., 1991). It is optimally active at 70°C and pH 5.0. A chromosomal DNA fragment with a length of 2025 bp carrying the structural gene coding for *T. thermosaccharolyticum* GA has been cloned and sequenced (Ducki et al., 1998). Homology up to 97% was found between the deduced amino acid sequence of GA from *T. thermosacharolyticum* and *Clostridium sp.*

G0005 (Ducki et al., 1998). They share some unique features, namely the absence of several helices that are present in eukaryotic GAs, with the GA from *Methanococcus jannaschii* (Coutinho and Reilly, 1997).

Study of the structures and enzymatic mechanisms of bacterial GAs has several advantages over that of their eukaryotic counterparts: 1) genetic manipulation is easier, and this makes the identification of structural and mechanistic roles of individual amino acid possible; 2) bacterial GAs are not glycoproteins, and have no sugar chains to complicate structural analysis of eukaryotic enzymes; and 3) the property differences of bacterial GAs from those well-investigated eukaryotic enzymes can provide more information about GA. These benefits are reinforced by a comparatively advanced state of understanding of the structures and mechanisms of eukaryotic GAs.

As a consequence of growth at high temperature and unique macromolecular properties, thermoanaerobic bacteria can often possess faster metabolic rates and more thermostable enzymes than can mesophilic microorganisms (Zeikus, 1979). In this research, the bacterium *T. thermosaccharolyticum* ATCC 7956 (also named *T. thermosaccharolyticum* DSM 571) was used to produce a prokaryotic GA. It is a strictly anaerobic bacterium and stringent anaerobic cell culture methods should be used.

Anaerobic fermentation

Human have used anaerobic fermentations since ancient times for many important fermentations such as ethanol production from yeasts, lactic acid preservation of foods, and anaerobic digestion of polysaccharides and proteins in ruminant cultivation and waste treatment. Anaerobic fermentations have contributed greatly to the development and success of industrial microbiology. Many ancient and contemporary industrial processes involve anaerobic fermentations. In recent years, the discovery of exotic and diverse anaerobic habitats

such as deep-sea thermal vents has led to the isolation of anaerobic organisms of biotechnological potential.

Anaerobic microbiology was considered a difficult area for a very long time. Many potential anaerobic bacteriologists were deterred from studying these diverse and interesting organisms because of the perceived difficulties in culturing them. Anaerobic microorganisms do not use molecular oxygen in biosynthesis and are incapable of using oxygen as a terminal electron acceptor. Instead, they use a diverse array of organic and inorganic electron donors and acceptors in their energy metabolism (Thauer et al., 1977; Zehnder, 1988; Zehnder and Stumm, 1988). Because of the widely diverse metabolic patterns among the anaerobes, anaerobic fermentations have properties not found in aerobic processes.

Oxygen concentrations and potential E_h levels are critical in the cultivation of anaerobic bacteria. Many anaerobes are so sensitive to oxygen that they cannot be isolated by conventional methods, such as growth in thioglycollate medium or culture in anaerobe jars. Pioneering work by Hungate on the bacterial flora of the rumen using roll-tube methods facilitated the isolation of some of the most oxygen-sensitive anaerobes (Hungate, 1950, 1969). The advantages of this method are: 1) the media are prereduced before they are inoculated and 2) the organisms are protected from exposure to oxygen during growth and transfer.

In the past few decades, several systems for cultivating anaerobes have been proposed for use in the clinical microbiology laboratory, and these have stimulated interest in anaerobic bacteriology. The most commonly used anaerobic systems include the Hungate roll-tube, the GASPAK jar system, and the anaerobic chamber (glove box) method (Killgore et al., 1972). Among these three methods, the GASPAK system requires relatively little space (for a single jar) and is quite simple to use. However, the jar has to be opened periodically to examine the cultures and thus puts the colonies in risk of exposure to atmospheric oxygen. This problem can be overcome by using the glove box system and the roll-streak tube system, but the glove

box system is rather expensive and occupies more space. The roll-tube method is comparatively complicated, but it is very practical and cheap to use.

In this research, the roll-tube method has been adopted to cultivate the strictly anaerobic bacterium *T. thermosaccharolyticum*. In essence, this method involves using prereduced and anaerobically sterilized (PRAS) medium, which is prepared in stoppered roll tubes. Exposure of bacteria and culture media to oxygen is avoided by displacing the air in culture tubes with an oxygen-free gas such as carbon dioxide or argon. When the stoppers are removed from the tubes for culture manipulation, anaerobiosis in the tubes is maintained by continuous flushing with oxygen-free gas.

GA purification and crystallization

Only proteins of high purity are easily characterized and crystallized. Different methods such as hydrophobic interaction, ion exchange, and size exclusion chromatography have been used to purify GAs from various sources (Okada, 1983; Mahajan et al., 1983; Bhella and Altosaar, 1984). Purification of bacterial GAs is particularly difficult due to their extremely low expression levels in the fermentation broth. Multiple chromatographic steps are usually required, in turn reducing GA yield. An affinity chromatography method has been developed to purify GA based on the high affinity of GA with its specific inhibitor acarbose (Clarke and Svensson, 1984). This method provides a rapid and efficient way to purify GA, especially when it is in low concentration in the stream to be purified.

With the development of biotechnological techniques and macromolecular engineering, there is an increasing need to elucidate the three-dimensional structure of proteins, nucleic acids and multi-macromolecular assembles by X-ray methods. To achieve this goal, crystals of good quality that suit high-resolution X-ray diffraction analysis must be obtained from macromolecular substances, always a hard task. Although some proteins may be very simple to crystallize, many others are elusive and stubborn (McPherson, 1989).

The reason that the crystallization step is the primary obstacle to expanded structure knowledge lies in its highly empirical nature (McPherson, 1976, 1982, 1985). Macromolecules are extremely complex physical-chemical systems whose properties vary in terms of many environmental factors such as pH, temperature, ionic strength, contaminants, and solvent composition. Effective manipulation of these factors to obtain good crystals requires much previous experience in similar situations. The strategy of all reported efforts for crystallization of biological macromolecules is to guide the system very slowly toward a state of minimal solubility by modifying solvent properties through equilibration with precipitating agents or by altering some physical property, like pH, to achieve a limited degree of supersaturation (McPherson, 1999). Among all these methods, vapor diffusion methods are far more popular than any other approach. They are ideal for screening a broad spectrum of crystallization conditions, and can be scaled up to obtain large crystals suitable for X-ray diffraction analysis. Furthermore, they can be carried out economically and conveniently while using a number of different physical arrangements. Virtually all vapor diffusion trials are carried out on a microscale with mother liquor solution volumes from 2 to 20 μL. These small volumes can be easily manipulated with micropipettes.

The three-dimensional structure of GA

Crystallographic studies of GA have been conducted on a proteolytic fragment of GA from *A. awamori* var. *X100*, which consists of the first 470 residues (including the entire catalytic domain) formed by the natural action of fungal acid proteases (Aleshin et al., 1992, 1994). The amino acid sequence of this GA fragment is about 95% homologous with the corresponding regions of GAs from *A. awamori* and *A. niger* (Coutinho and Reilly, 1994a). The catalytic domain of this GA contains thirteen α-helices, twelve of which are arranged in pairs forming an $(\alpha/\alpha)_6$ barrel (Figure 2). The cavity in the center of the barrel is composed of highly conserved residues that are involved in the active site. In addition, homology anal-

Figure 2. Cartoon display (RasMol Version 2.6) of the catalytic domain of GA from *Aspergillus awamori* var *X100* (Aleshin et al., 1994).

ysis of thirteen amino acid sequences of GAs shows five conserved regions in the catalytic domain define the active site (Coutinho and Reilly, 1994a).

These five conserved regions can also be found in bacterial GAs (Ducki et al., 1998). Unlike the catalytic domain of fungal GAs (except that of *R. oryzae*), which is at their *N*-termini, in the *T. thermosaccharolyticum* DSM 572 GA the catalytic domain is at the *C*-terminus (amino acids 271 to 695). The comparison of the crystallographically determined (for *A. awamori var. X100*) and the calculated (for *T. thermosaccharolyticum*) secondary structures of the catalytic domain showed high similarities in the arrangement of the helices and β-sheets (Ducki et al., 1998). Further experiments demonstrated that GAs from bacteria and fungi share a common macromolecular architecture in their catalytic center, even though some helices existing in the peripheral parts of fungal GAs are missing in the bacterial GAs (Ducki et al., 1998). Evidently, the structural organization of the $(\alpha/\alpha)_6$ barrel of fungal GAs is not completely evolved in bacterial GAs. Nevertheless, bacterial GAs are still very efficient catalysts (Coutinho and Reilly, 1997; Ducki et al., 1998).

Research Objectives

At the time I chose this project, no bacterial GA had yet been crystallized. It appeared very interesting and valuable to crystallize this thermophilic GA and further solve its three-dimensional structure by X-ray crystallography. Therefore, the aim of this research involved six aspects:

1. Design anaerobic equipment and methodology to cultivate a thermoanaerobic bacterium.
2. Scale up the process and produce the GA in large fermentors.
3. Optimize the purification method to obtain the purified enzyme by fast protein liquid chromatography (FPLC).
4. Characterize this prokaryotic GA including its optimal pH, temperature, thermostability, amino acid composition, and *N*-terminal amino acid sequence.

5. Compare the properties of this GA with those of well-studied eukaryotic GAs.
6. Try to crystallize this enzyme and solve its three-dimensional structure by X-ray crystallography.

Especially I would mention here Dr. Alexander Aleshin, an associate scientist in the Department of Biochemistry, Biophysics, and Molecular Biology at Iowa State, who has collaborated with me to do the GA crystallization and X-ray crystallography. Equally, I would also like to mention Sonja Berensmeier, a Technical University of Braunschweig student, who carried out an internship during the spring and summer of 2000 in our laboratory and who helped to develop the purification method used to purify the GA from *T. thermosaccharolyticum* DSM 571.

MATERIALS AND METHODS

Experimental

The whole procedure to carry out this research can be seen in Figure 3. It generally includes anaerobic fermentation, GA recovery and purification, purified GA characterization and crystallization. Detailed information about materials and methods for each step will be given later.

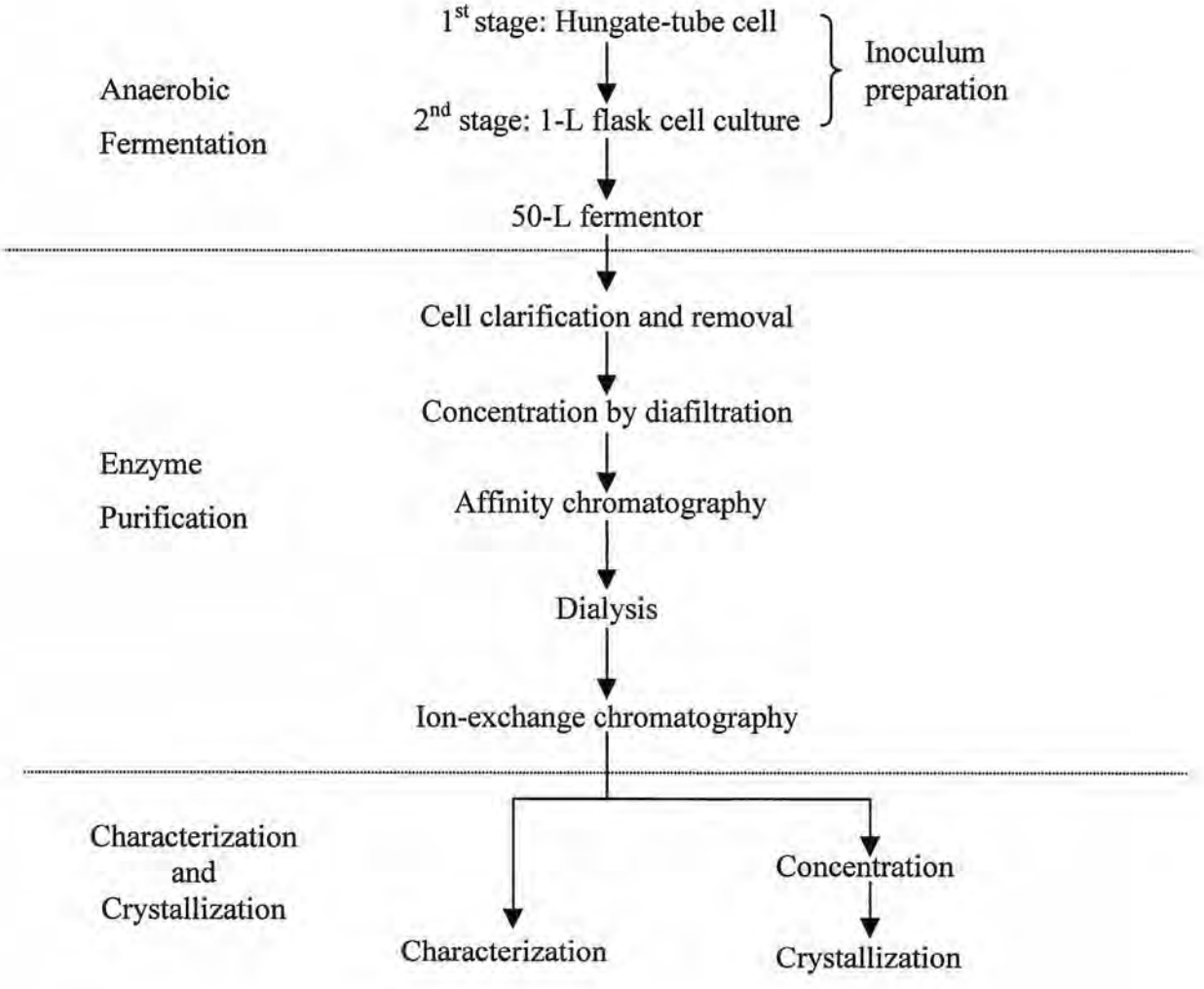

Figure 3. Flow chart of unit operations for this thesis.

Organism and Cultivation

T. thermosaccharolyticum ATCC 7956 (also called *T. thermosaccharolyticum* DSM 571) was obtained from the American Type Culture Collection (Manassas, VA). Strict anaerobic techniques (Hungate, 1950, 1969) were employed both for medium preparation and cultivation. The spores were first activated by culturing them in beef liver medium until obvious turbidity was observed, and then they were aseptically aliquotted to several tubes with sterile glycerol medium and stored at –70°C for future use. For growth experiments and enzyme preparation, the strain was cultivated anaerobically at 62°C in the following complex SYT medium containing (per L): 10 g potato starch, 5 g tryptone, 3 g yeast extract, 5 g beef extract, 10 g MES (2-[*N*-morpholino]ethanesulfonic acid), 10 mg $FeSO_4 \cdot 7H_2O$, 0.5 g cysteine hydrochloride, and 1 mg resazurin, with pH adjusted to 7.2 with 8 N NaOH. The culture medium was autoclaved for 45 min to ensure that extremely heat-resistant spores were killed. The flask or fermentor was inoculated with a 2% volume of an early log-phase inoculum, which was previously subcultured in the same medium using 15-mL Hungate tubes or flasks. The fermentor was agitated at 120 rpm and gassed continuously with argon. Samples of 15 mL were withdrawn at appropriate times and centrifuged for 20 min at 5000 x *g* for analysis.

Anaerobic Method

Preparation of oxygen-free gas

Oxygen-free gas is essential to culture fastidious anaerobes. Commercially obtained gas is often contaminated with traces of oxygen, which may be removed by passing it through a column of hot reduced copper turnings. Figure 4 shows the equipment I designed for our laboratory, which was very satisfactory for this process. The heated copper turnings need to be reduced periodically by passing hydrogen through them.

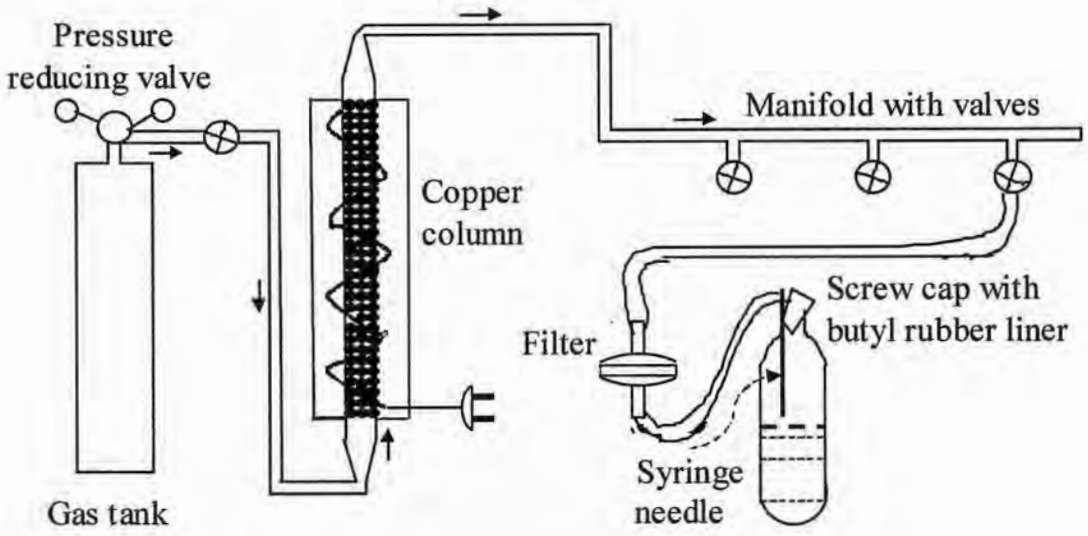

Figure 4. Apparatus for maintaining an anaerobic atmosphere in PRAS medium.

Preparation of anaerobic media (Hungate, 1950, 1969)

1. Weigh out the dry ingredients according to the above recipe and place in a conical flask.
2. Add distilled water and resazurin (oxygen indicator) to 1 mg/L final concentration.
3. Place a polypropylene magnetic stirring bar in the flask, fit a chimney to the flask, and heat the solution on a flat-top heater while stirring.
4. Boil the medium to drive off the dissolved oxygen until the color of the resazurin indicator turns from pink to almost colorless (original medium color). Remove the flask from the heat and remove the chimney. Immediately pass a stream of oxygen-free argon through the medium; the flow rate should be sufficient enough to cause gentle bubbling.
5. Cool the medium and add the reducing agent L-cysteine hydrochloride (0.05 g/L).
6. While continuously flushing the gas, adjust the pH of the medium to the desired value.
7. Anaerobically dispense medium into tubes that are being flushed with oxygen-free argon. Close the culture tube with a butyl rubber liner and screw cap.
8. Sterilize tubes of medium in the autoclave at 121°C for 45 min.

9. Store tubes at room temperature for future use. Discard tubes if media color becomes red.

Localization of Enzyme

To find out whether GA is an intracellular or extracellular product, 400 mL culture broth from the stationary phase (40 h) was collected. Cells were separated from broth by centrifugation at 8,000 x *g* for 25 min. GA activity in the resulting supernatant was measured. The cell pellet (3.6 g wet weight) was washed twice with 0.05 M sodium acetate buffer, pH 4.5. The washed cells were suspended in 10 mL of the same buffer and disrupted in a French press at 80 MPa. Cell debris was removed by centrifugation at 15,000 x *g* for 30 min and the supernatant was then used to determine the intracellular GA activity. The cell debris was washed again with the above buffer and centrifuged at 15,000 x *g* for 30 min, so that additional intracellular GA possibly trapped in the cell debris could also be determined.

Enzyme Assay

GA activity was determined at 60°C with 2% soluble potato starch as substrate in 0.05 M sodium acetate, pH 4.5. Aliquots of the assay mixture were quenched at several time points by addition to 4 M Tris-Cl, pH 7.0, to a final concentration of around 1.1 M Tris. Glucose was determined in the quenched reaction mixtures by the glucose oxidase-peroxidase method in which oxidized *o*-dianisidine formed is proportional to the amount of glucose present. A unit of activity is defined as the amount of enzyme that produces 1 μmol/min of glucose under the above assay conditions.

Protein Assay

Protein concentration was determined with the BCA (bicinchoninic acid) protein assay reagent (Pierce) by using bovine serum albumin as the protein standard. This method is based on the reaction of Cu^{2+} with peptides in alkaline solution to form Cu^{+} ions, which can be detected by a highly sensitive and selective BCA reagent. GA sample was diluted if necessary

to less than 2.0 mg/mL before a 0.1-mL sample was taken out and incubated with 2.0 mL assay mixture at 37°C for 30 min. The absorbance was measured at 562 nm, and the protein concentration was read from a standard curve prepared in the same way with BSA.

Preparation of Acarbose-Sepharose Affinity Resin

Step 1: Preparing the gel

1. Weigh out 10 g freeze-dried powder of CNBr-activated Sepharose® 4 Fast Flow (Amersham Pharmacia Biotech) and suspend in 15 mL of 1 mM cold HCl solution.
2. The gel swells immediately and is then washed for at least 15 min with approximately 2 L of 1 mM HCl (added in several aliquots) on a sintered glass filter. About 45 mL drained gel was obtained for the next step.

Step 2: Coupling the ligand

1. Dissolve 1.5 g acarbose in 50 mL coupling buffer: 0.1M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl.
2. Mix the coupling solution containing the acarbose with the prepared gel in a stoppered bottle. Rotate the mixture end-over-end for 3.5 h at room temperature.
3. Wash away excessive ligand with at least 200 mL coupling buffer.
4. Block any remaining active groups by transferring the gel to 0.1M Tris-HCl buffer, pH 8.0. Let it stand for 2.5 h.
5. Wash the product with at least three cycles of alternating pH. Each cycle consists of a wash with 200 mL of 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl, followed by a wash with 200 mL of 0.1 M Tris-HCl buffer, pH 8.0.

The resin is now ready for use or storage in 0.02 % sodium azide solution for future use.

Protein Purification

Sample preparation

For the preparation of large amounts of GA, fermentations were carried out in a B. Braun 50-L fermentor (ISU Fermentation Facility) inoculated with a 1-L (2% (v/v)) growing *T. thermosaccharolyticum* culture. At the end of the fermentation, the cells were removed continuously by an Amicon Diaflo® hollow-fiber cartridge with a 0.1-μm cutoff (ISU Fermentation Facility). This cell-free supernatant (48 L) was then concentrated to 2.5 L by an ultrafiltration device (H1P10-43, 10 kDa cutoff, Amicon), and diafiltered against 0.1 M NaOAc/0.5 M NaCl buffer, pH 4.5, to give a final volume of 2.45 L.

Column chromatography

GA purification was carried out by using FPLC on Biologic Systems (Bio-Rad) and Äkta Explorer (Amersham Pharmacia Biotech) systems. Of the above sample, 1.45 L was loaded at 5 mL/min to a 32-mL acarbose-Sepharose affinity column previously equilibrated with the above buffer. After the column was washed with the same buffer until the absorbance reached the baseline value, the enzyme was eluted at 1.0 mL/min with 1.7 M Tris-Cl, pH 7.6. Fractions (6 mL/tube) were collected and tested for GA activity. Fractions with high activity (>1500 U/L) were pooled together and dialyzed against 30 mM Tris-Cl, pH 7.5, before loading onto the anion-exchange column. Q-Sepharose Fast Flow resin (80 mL, Sigma) was equilibrated with 30 mM Tris-Cl buffer (pH 7.5) and the bound proteins were eluted at a flow rate of 1 mL/min with a linear gradient (0 to 500 mM) of NaCl (360 mL). Fractions (6 mL/tube) with high GA activity were pooled and processed as needed for future analysis.

Electrophoresis and Molecular Weight Determination

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out in 4–20% Tris-HCl Mini-PROTEAN II Gradient Ready gels (Bio-Rad). Samples in 2%

SDS–5% β-mercaptoethanol were previously heat-treated at 100°C for 5 min. Electrophoresis was run at room temperature for about 50 min at 200 V. Protein bands were visualized by staining the gel with Coomassie Blue R-250 solution. Prestained SDS-PAGE standards with molecular weights from 7,400 to 203,000 Da (Broad Range, Bio-Rad) were used to estimate the molecular weight of the purified GA.

Protein Transfer Blotting

For protein sequencing and amino acid analysis, the proteins were electro-transferred to a chemically stable membrane, polyvinylidine difluoride (PVDF) after being separated by SDS-PAGE. The gel was equilibrated in transfer buffer without methanol (25 mM Tris, 192 mM glycine) for 20 min instead of being stained. Meanwhile the PVDF membrane was activated by dipping in methanol followed by soaking in transfer buffer containing methanol for another 20 min. The membrane was then carefully put on the top of a filter paper, which had been saturated with the same transfer buffer without ethanol. The gel was then put on top of the PVDF membrane, any trapped air bubbles were removed, and it was covered by another filter paper treated the same way. This gel-PVDF sandwich was placed in a semi-dry gel transfer unit (Bio-Rad) for 30 min at 10 kV to complete the protein transfer from gel to PVDF membrane. After transfer, the membrane was stained with Coomassie Blue R-250 and destained to visualize the protein bands. Sections containing the protein bands can be excised for amino acid analysis and *N*-terminal protein sequencing.

Amino Acid Analysis

The amino acid composition of the purified GA was determined by an Applied Biosystems 420A Derivatizer/120A Analyzer (ISU Protein Facility) after sample was hydrolyzed with 6 N HCl at 150°C for 65 min.

N-Terminal Amino Acid Sequencing

The bands were cut from the PVDF membrane, washed six times with deionized water, and loaded onto a pre-conditioned filter for sequence analysis. The *N*-terminal amino acid sequence of the purified GA was determined by stepwise Edman degradation with an Applied Biosystems 494 Procise Protein Sequencer/140C Analyzer (ISU Protein Facility).

Effect of pH and Temperature on GA Activity

To find the optimal conditions for enzyme activity, the effect of both pH and temperature were investigated. The effect of pH on the activity of purified GA was measured at different pHs from 3.0 to 8.0 at 60°C using 50 mM sodium citrate (pH 2.5–4.0), 50 mM sodium acetate (pH 4.0–6.0), or 50 mM sodium phosphate (pH 6.0–8.0). The effect of temperature on GA activity was measured in the range of 30°C~80°C in 50 mM sodium acetate, pH 5.0. The GA concentration used in these assays was 4.0 μg/mL.

Thermostability

To estimate enzyme thermostability, purified GA was incubated in 50 mM sodium acetate buffer (pH 5.0) at several temperatures from 60~80°C for various periods of time. Samples were removed at 1-h time intervals and chilled on ice immediately, then the residual GA activity was measured using the enzyme assay.

Effect of Metal Cations and Other Chemicals on GA Activity

The effect of cations on GA activity was investigated by incubating pure GA with Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, and Pb^{2+} at final concentrations of 0.1 mM, 1 mM, and 5 mM for 5 min at 60°C, then assaying for activity. The effect of EDTA and dithiothreitol on GA activity was determined similarly at final concentrations of 1, 5, and 10 mM, respectively.

Determination of the Free Sulfhydryl Groups of GA

Free sulfhydryl (–SH) groups on the protein surface will influence protein properties and crystallization. They can be modified by 5,5'-dithiobis(2-nitrobenzoate) (DTNB), which forms thionitrobenzoate with the protein and liberates 1 mol thionitrobenzoate anion for each –SH group. This colored anion can be determined from its absorption at 412 nm ($\varepsilon = 1.36 \times 10^4\ M^{-1}cm^{-1}$ at pH 7.5). Procedures for determining total number of free –SH groups per mol pure GA are as follows:

1. Add 10 μL of stock DTNB solution (39.6 mg in 10 mL of 0.1 M phosphate buffer, pH 7.0) to 1.0 mL of purified GA (concentration around 0.5 mg/mL).
2. The reaction mixture is incubated for 2~3 min at room temperature.
3. Read the absorbance at 412 nm, compared with a blank containing 30 mM Tris-Cl (pH 7.5) instead of GA ($\varepsilon = 1.36 \times 10^4\ M^{-1}cm^{-1}$).
4. Calculations:

$$\text{Mols of free (–SH)} = \frac{Abs_{sample} - Abs_{blank}}{1.36 \times 10^4}$$

$$\text{Number of free (–SH) per mol GA} = \frac{\text{Mol free –SH}}{\text{Mol GA}}$$

Crystallization – Vapor Diffusion in Hanging Drops

The hanging drop method, suited to screen a large number of crystallization conditions with a small quantity of material required, was adopted in this study, and the vapor phase was used to bring the the system into equilibrium. Purified GA was first concentrated to about 15–20 mg/mL by ultrafiltration with a 10 kDa cut-off membrane or Centriprep™ concentrators (Amicon). With this crystallization approach, a protein microdroplet (as small as 2 μL) of

mother liquor was suspended from the underside of a microscope cover slip that had been siliconized to prevent wetting and drop spreading, and then subsequently placed over the well of a plastic plate. Prior to operation the individual wells had been prefilled with 0.5 mL of precipitating solution. These plates were stored at room temperature and crystals could be easily examined by putting the plates under a dissecting microscope.

Chemicals, Reagents, and Equipment

Unless otherwise specified, all reagents were purchased from Sigma. Acarbose was donated by Jayarama Shetty of Miles Laboratories. All other chemicals were of analytical reagent grade. Ready gels and protein markers were bought from Bio-Rad. Membranes and hollow fiber unit for concentrating protein solutions were purchased from Millipore.

RESULTS AND DISCUSSION

Growth Characteristics

Several culture media and conditions were tested to optimize the production of GA from *T. thermosaccharolyticum* ATCC 7956. They were 1) beef liver medium (Appendix A) cultured at 62°C; 2) medium that had been applied to cultivate *T. thermosaccharolyticum* ATCC 27384 (Appendix A) at pHs 6.7 and 7.2; and 3) SYT medium at pHs 6.6 and 7.2. Based on the final GA activity level, the current medium of SYT at pH 7.2 and temperature at 62°C (Materials and Methods) was a better choice to cultivate *T. thermosaccharolyticum* ATCC 7956. Figure 5 shows cell growth, pH change, and GA production during a 46-h fermentation in a 50-L fermentor. With starch as the sole carbon source, this strain had a fairly long lag phase, almost 12 h, when a slight increase of cell density (OD_{580}) and a drop of pH from 7.0 to 6.8 occurred and no GA activity was detected. After this adaptation period come a quick cell growth phase lasting about 15 h, with an almost tripling of cell density. With rapid medium consumption and product formation to support the cell growth and energy maintenance, the broth pH dropped quickly from 6.8 to 5.6. Meanwhile, GA was secreted into the fermentation broth in limited amounts, with less than 30 U/L being detected at the end of this phase. Much more GA was produced extracellularly during the stationary and death phases, reaching a maximum activity of about 170 U/L after a 44-h fermentation. The behavior of this strain is different from *T. thermosaccharolyticum* DSM 572, which gave final activity of about 30 U/L after 24-h fermentation period (Specka et al., 1991).

Enzyme Localization

Localization of GA activity was studied according to the flow chart presented in Figure 6. The total extracellular activity in a 400-mL culture broth (40 h) after removal of cells by centrifugation was 56 U. The GA activity in the supernatant obtained after disruption of the cells (3.6 g wet weight) was 0.98 U. Membrane-bound activity was not determined. Since

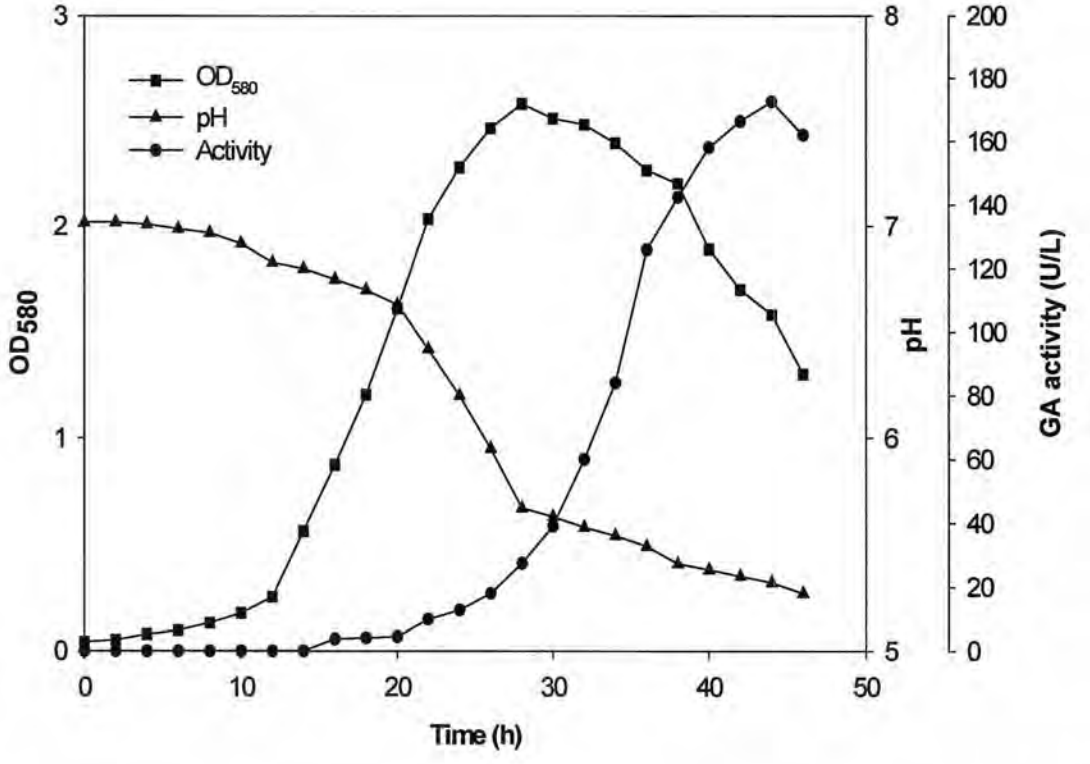

Figure 5. Production of GA during growth of *T. thermosaccharolyticum* ATCC 7956 on 2% (w/v) starch at 62°C under anaerobic conditions. One liter of growing inoculum (OD_{580} = 2.54) was inoculated into a 50-L fermentor. Samples were taken at 2-h intervals for analysis.

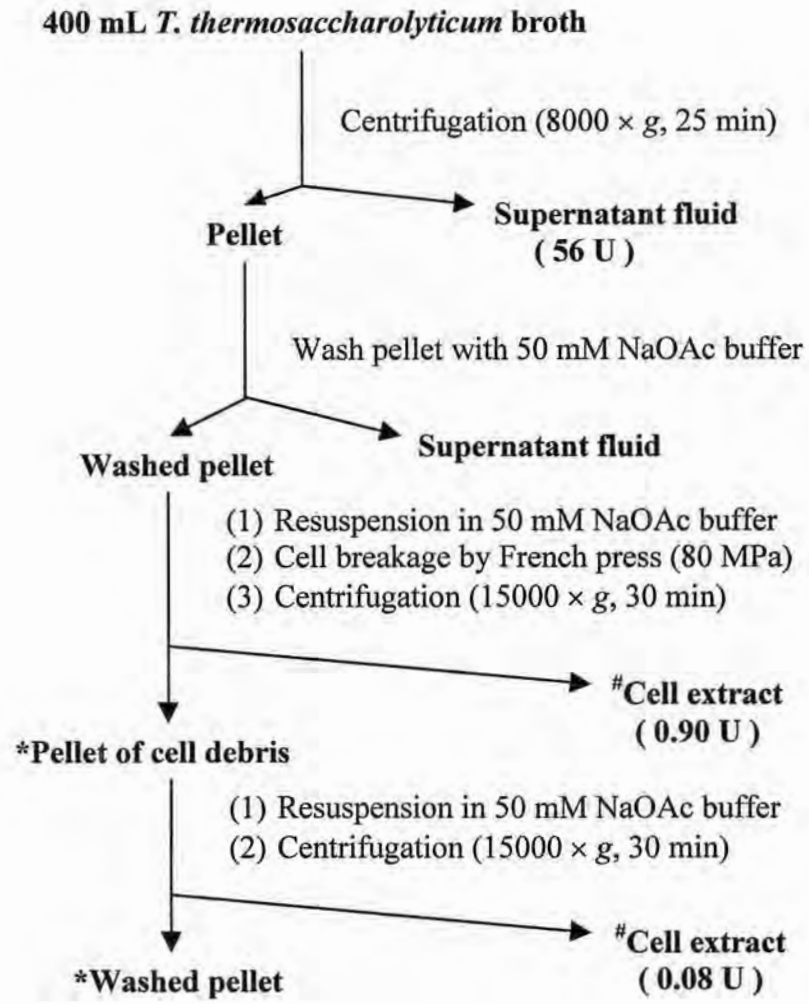

Figure 6. Localization of GA activity in *T. thermosaccharolyticum* ATCC 7956 (*membrane-bound activity; #intracellular activity).

most of the GA is secreted to the fermentation broth, all later studies of GA were based on this extracellular product if not otherwise specified.

GAs produced by strains *Clostridium sp. G0005* and *C. thermosaccharolyticum* DSM 572 resided on the cell surfaces (Ohnishi et al., 1992; Specka and Mayer, 1993). However, about 50% of the total GA activity was found in the extracellular culture broth of these organisms. GA localization varies with strains and growth conditions, and this may explain the higher extracellular GA activity produced by *T. thermosaccharolyticum* ATCC 7956 in this study.

GA Purification

A four-step combination of polyethylene glycol 400 precipitation, Q-Sepharose anion exchange, hydroxyapatite adsorption, and Superdex size exclusion chromatography was reported in GA purification from *C. thermosaccharolyticum* DSM 572 broth and gave 63-fold purification with 36% total recovery (Specka et al., 1991). Similar purification methods except using Sephacryl S-300 HR instead of Superdex 200 in the SEC step were tried on this *T. thermosaccharolyticum* ATCC 7956 GA, but they gave very low resolution and purification. Therefore the acarbose-Sepharose affinity chromatography followed by a step of anion exchange chromatography was chosen as the final purification strategy, and this showed a marked improvement in GA purification.

The crude enzyme sample obtained from concentration and diafiltration against 0.1 M NaOAc/0.5 M NaCl buffer contained 950 U/mL of GA. A 1.45-L sample was first loaded on an acarbose-Sepharose affinity column, unbound and weakly bound proteins were washed out with 15 column volumes of equilibrium buffer, then the tightly bound proteins including GA were eluted by 1.7 M Tris-HCl buffer (pH 7.6) in 6 column volumes. The complete chromatogram is shown in Figure 7. Not surprisingly, most proteins did not interact with acarbose, a GA inhibitor, and flowed through the column without binding, as indicated by the

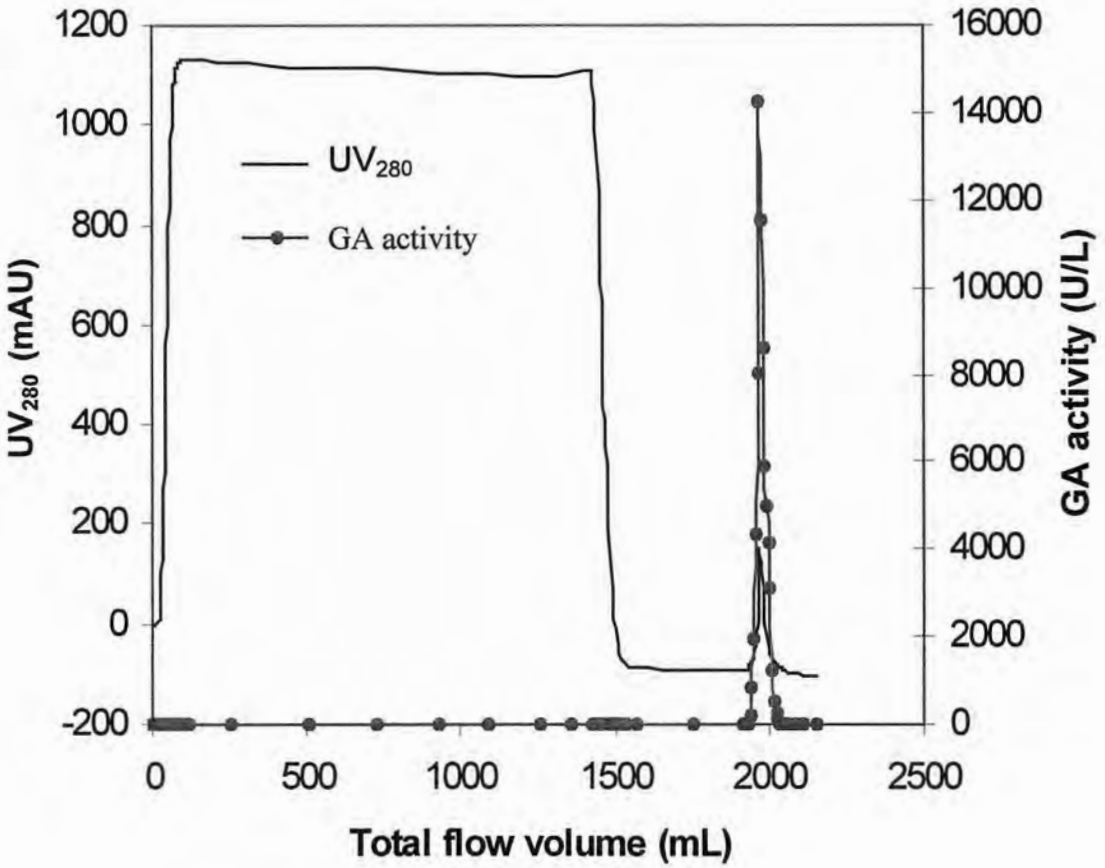

Figure 7. Purification of *T. thermosaccharolyticum* ATCC 7956 GA on the acarbose-Sepharose affinity column. Concentrated supernatant (1.45 L) was first loaded onto the column at 6 mL/min, after washing to the baseline with the equilibrium buffer (0.1 M NaOAc/0.5 M NaCl, pH 4.5), GA was then eluted at 1.5 mL/min with 1.7 M Tris-HCl, pH 7.6. All eluted fractions (6 mL/tube) were collected and tested for enzyme activity. Fractions 5 to 14 with high GA activity were pooled together (fraction number not shown).

large initial peak in Figure 7. Elution with 1.7 M Tris-HCl at pH 7.6 resulted in a single small sharp peak, which showed high GA activity. By this step alone, a specific activity of 39.2 U/mg was achieved, which is 245-fold higher than that of the original sample (Table 1).

However, besides the dark band corresponding to GA molecular weight, there were still several other visible bands on the SDS-PAGE gel (Figure 8), so Q-Sepharose anion exchange chromatography was used to further purify the GA. During this ion-exchange step, GA bound to the Q-Sepharose in 30 mM Tris-HCl buffer (pH 7.0) and then was desorbed from the column by a linearly increasing NaCl concentration (Figure 9). A single GA peak was obtained between 0.17 to 0.24 M NaCl. A protein adsorption platform was observed prior to the GA elution, implying the existence of contaminants that more weakly bind to Q-Sepharose than does GA. SDS-PAGE analysis of the GA peak confirmed the removal of those light bands that appeared in the affinity-purified sample, as only a single protein band was finally obtained with a molecular weight of about 77 kDa (Figure 8). The overall two-step purification procedure yielded a pure GA fraction with a specific activity of 49.4 U/mg and with a total purification and recovery of 309-fold and 25.8%, respectively. A complete summary of the purification is given in Table 1.

Apparently, this two-step method is superior to the earlier four-step method by giving a much higher purification capability and a similar recovery. There is not much GA activity loss in the second purification step of anion exchange, although more than 70% of the total activity was lost in the affinity chromatography step. This might be caused by the following factors: 1) irreversible interaction of GA with acarbose; 2) denaturation and/or inactivation of GA under the loading/washing/elution conditions in the affinity column. Thus, further optimization of the affinity purification step would be helpful to increase the overall yield. The problem of activity loss was also reported in purification of *A. awamori* GA when using acarbose-Sepharose affinity column (Chen et al., 1993).

Table 1. Purification of GA from *T. thermosaccharolyticum* ATCC 7956[a]

Purification step	Volume (ml)	Activity (U/L)	Total activity (U)	Total protein (mg)	Specific activity[d] (U/mg)	Yield[e] (%)	Purification factor[f]
Concentrated supernatant	1450	950	1377.5	8604	0.16	100	1
Affinity chromatography	60[b]	6693[c]	401.6	10.25	39.18	29.2	245
Anion-exchange chromatography	57[b]	6225[c]	354.8	7.18	49.42	25.8	309

[a] After growth of the culture in a 50-L fermentor for 42 h, the culture supernatant was concentrated to 2.45 L, and then 1.45 L was used for enzyme purification.

[b] Combined volume of high-activity fractions.

[c] Average activity of the above combined fractions.

[d] Specific activity is defined as enzyme units per mg of total protein in the same sample.

[e] Yield is the percentage of total GA activity in the purified fraction pool to the total loaded activity.

[f] Purification factor is the ratio of the specific activity of the purified fraction pool to that in the original sample.

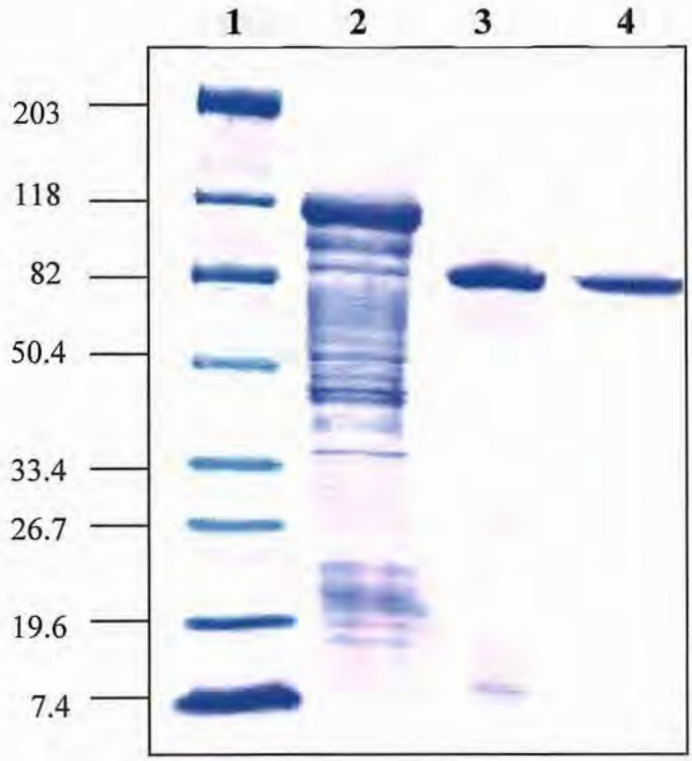

Figure 8. SDS-PAGE of GA from different purification steps on 4-20% Tris-HCl ready gel (Coomassie blue stain). Lanes: 1) Prestained standard proteins, with molecular weight in kDa (top to bottom): myosin (203), β-galactosidase (118), bovine serum albumin (82), ovalbumin (50.4), carbonic anhydrase (33.4), soybean trypsin inhibitor (26.7), lysozyme (19.6), and aprotinin (7.4); 2) concentrated supernatant; 3) after acarbose-Sepharose affinity column; 4) after Q-Sepharose Fast Flow column.

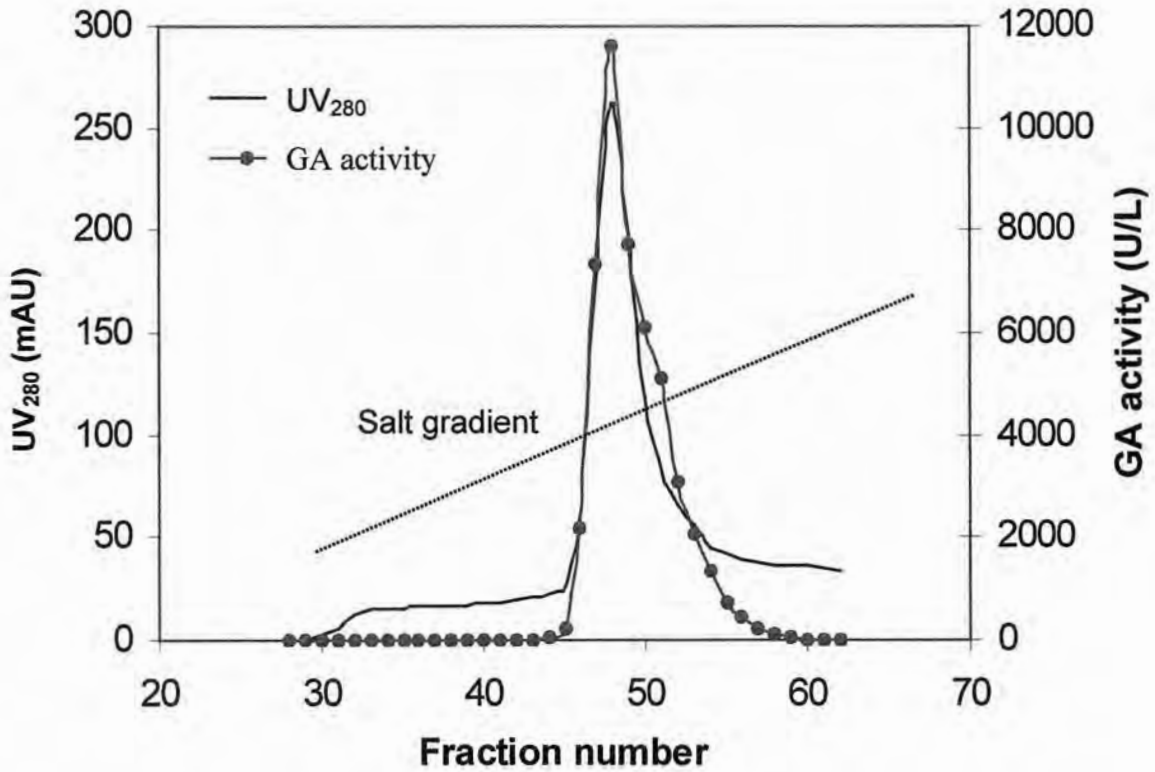

Figure 9. Purification of GA from affinity chromatography on a Q-Sepharose Fast Flow column. Of the pooled sample, 60 mL was loaded at 1 mL/min to the ion-exchange column that was previously equilibrated with 30 mM Tris-HCl, pH 7.5. Elution was performed with a 360-mL linear salt gradient (0~500 mM NaCl) in equilibrium buffer at a flow rate of 1 mL/min. Fractions (6 mL/tube) 46 to 55 with high GA activity were collected.

Amino Acid Composition and *N*-Terminal Sequence

The amino acid composition of GA from *T. thermosaccharolyticum* ATCC 7956 is presented in Table 2. The sequence of the first fifteen *N*-terminal amino acid residues is Val-Leu-Ser-Gly-Lys-Ser-Asn-Asn-Val-Ser-Ser-Ile-Lys-Ile-Asp. This sequence is identical to that of GAs from *Clostridium sp. G0005* and *T. thermosaccharolyticum* DSM 571 and 572, but has low similarity with that of other fungal GAs (Ducki et al., 1998).

Dependence of pH and Temperature on GA activity

The purified *T. thermosaccharolyticum* GA is active in a broad pH range between 3.5 and 6.5, with optimal activity at pH 5.0 (Figure 10), and from 50 to 70°C with optimal activity at 65°C (Figure 11a). These properties are similar to most reported prokaryotic GAs (Specka et al., 1991; Ohnishi et al., 1991; Ganghofner et al., 1998); however, they are different from fungal GAs, which exhibit pH optima between 4 and 5 and temperature optima of 40~60°C (Manjunath et al., 1983).

The activation energy of *T. thermosaccharoyticum* GA from 30 to 60°C is 38.7 kJ/mol (Figure 11b), which is lower than that of *A. niger* GA I (59.9 kJ/mol or 67.4 kJ/mol) (Meagher and Reilly, 1989; Olsen et al., 1992) or GA II (62.2 kJ/mol) (Meagher and Reilly, 1989).

The thermostability of this GA at pH 5.0 was studied at several temperatures from 60°C to 80°C (Figure 12). It was very thermostable at temperatures up to 70°C. There was almost no activity loss during 6 h incubation at 60°C and 70°C. However, incubations at temperatures beyond 70°C resulted in rapid activity loss. As illustrated in Figure 12, almost 86% and 100% of the original GA activity was lost in the first hour of incubation at 75°C and 80°C, respectively, and no detectable activity was left after 3 h at 75°C. Most fungal GAs are quite stable at temperatures below 60°C, but can be rapidly inactivated above 60°C (Manjunath et al., 1983).

Table 2. Amino acid composition of GA from *T. thermosaccharolyticum* ATCC 7956

Amino acid	pmol	Residules/mol[a] (Nearest integer)
Aspartic acid	1016	138
Glutamic acid	463	63
Serine	340	46
Glycine	425	58
Histidine	69	9
Arginine	222	30
Threonine	240	32
Alanine	342	46
Proline	189	26
Tyrosine	361	49
Valine	159	22
Methionine	47	6
Isoleucine	228	31
Leucine	247	33
Phenylalanine	117	16
Lysine	585	79
Cysteine	ND[b]	ND[b]
Tryptophan	ND[b]	ND[b]

[a] The number of residues per mole is based on the estimated molecular size of 77 kDa.

[b] ND, not determined.

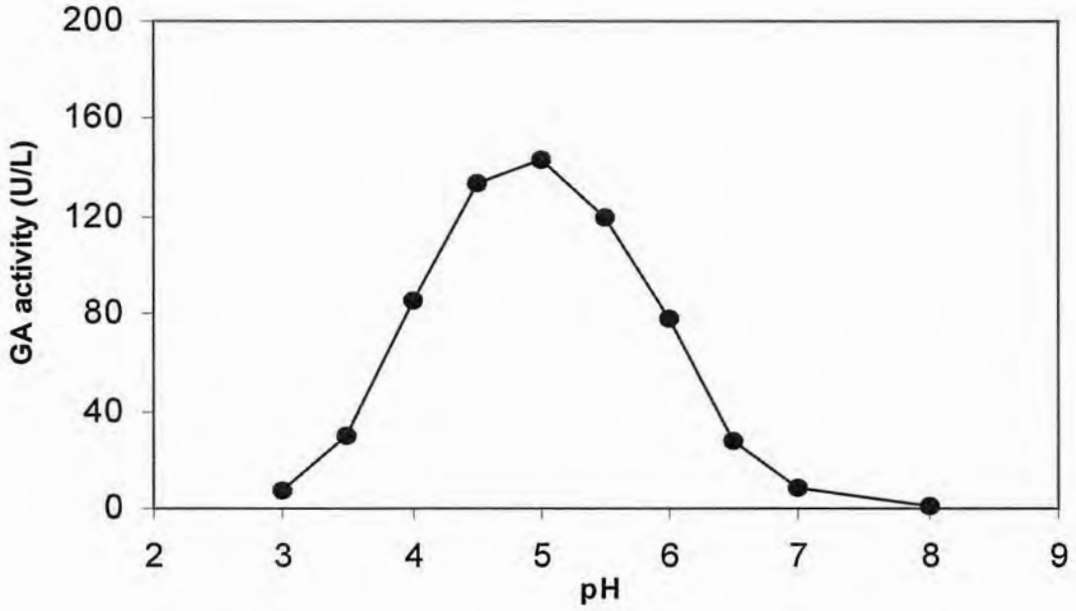

Figure 10. Effect of pH on GA activity of *T. thermosaccharolyticum* ATCC 7956. Enzyme activity was assayed in buffers with different pHs from 3.0 to 8.0 at 60°C using 2% starch as substrate.

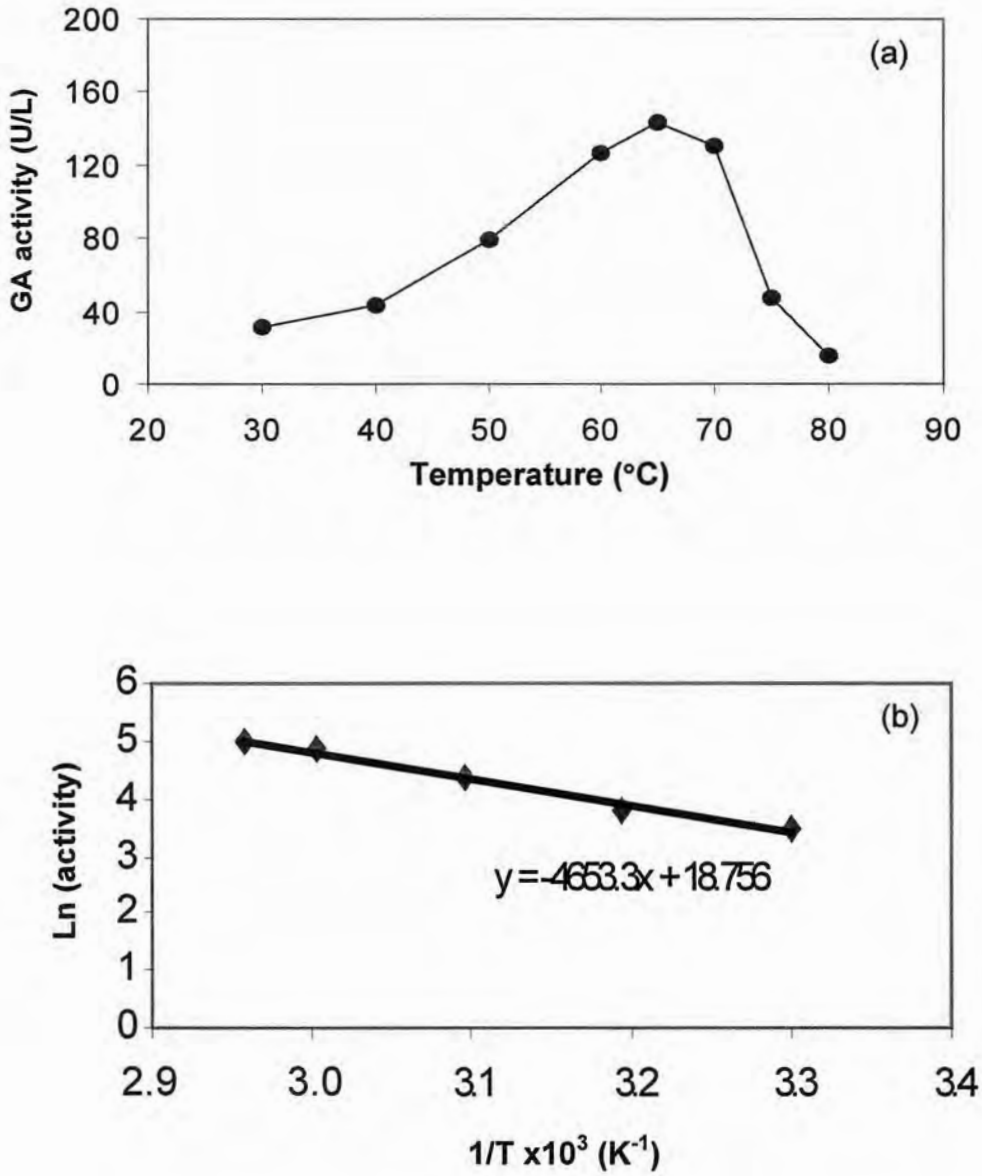

Figure 11. Effect of temperature on GA activity of *T. thermosaccharolyticum* ATCC 7956. Enzyme activity was assayed in 50 mM sodium acetate buffer (pH 5.0) at different temperatures using 2% starch as substrate.

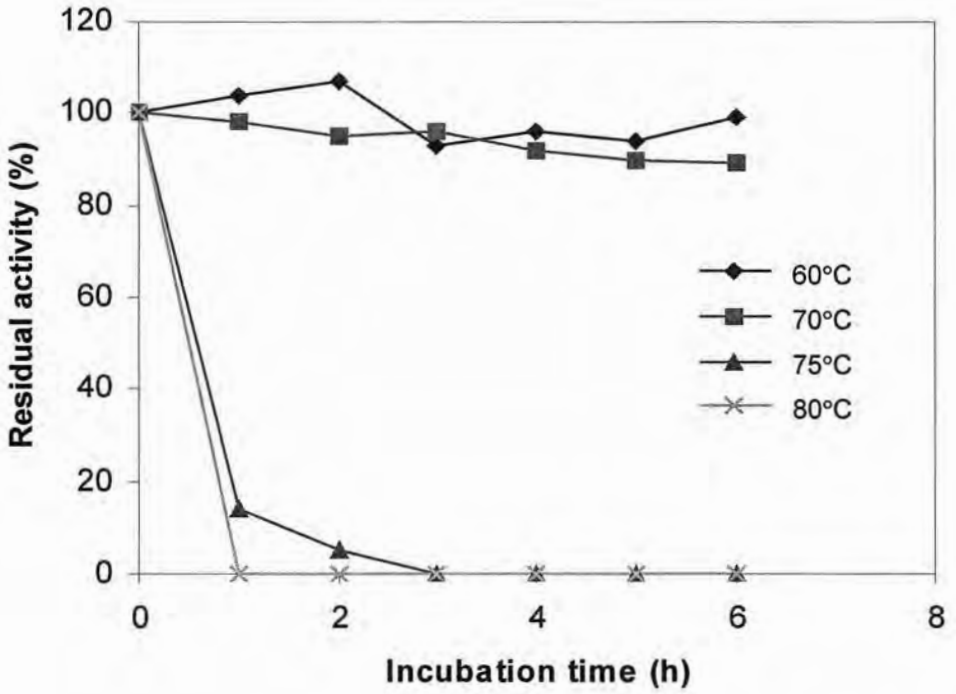

Figure 12. Thermostability of *T. thermosaccharolyticum* ATCC 7956 GA. Purified GA was incubated in 50 mM sodium acetate buffer (pH 5.0) at 60°C~80°C, and residual GA activities were measured at 1-h intervals. Full activity corresponds to 40 U/mg of protein as GA.

Effect of Metal Cations and Other Chemicals on GA Activity

Ca^{2+} and Mg^{2+} did not influence GA activity up to 5 mM, nor did Mn^{2+} at 1 and 5 mM, respectively (Table 3). Co^{2+} at 5 mM inhibited GA activity by 35%. Zn^{2+}, Cu^{2+}, and Pb^{2+} inhibited GA activity at 1 mM by 59, 47, and 39%, with their relative activities dropping to less than 20% at 5 mM. Neither the metal chelator EDTA nor the anti-oxidant DTT showed much inhibition of GA activity at 10 mM.

No metal ions seem to be required for the activity of this *T. thermosaccharolyticum* GA, which also held true for most of the fungal GAs (Saha and Zeikus, 1989a). Differences of inhibition on some fungal GAs were also reported (Takahashi et al., 1981; El-Abyad et al., 1994).

Crystallization

Many crystallization screening matrices have been investigated on several samples from different purification protocols. Although crystals have been formed under some conditions, they do not have smooth edges and sometimes are badly twinned. The best conditions to crystallize this GA are 15% PEG 3350, 100 mM Tris-HCl, and 200 mM Li_2SO_4 at pH 8.0. Under these conditions, crystals grow to dimensions of 0.2 x 0.2 x 0.02 mm, which is suitable for X-ray data collection. All these crystals were taken to Brookhaven National Laboratory for diffraction. Although they diffracted, few were suitable for X-ray analysis. The general problem is that the crystals were badly disordered in one direction. As a result, the resolution in that direction was about 3 Å, while in other directions it was better than 2 Å. The nonuniformity of the diffraction makes molecular replacement unlikely to solve the structure at present. Purification protocols may influence the protein structure, but how it works is still not known. Different purification methods have been tried, including hydroxyapatite and gel filtration, but they gave low resolution and purification. Therefore, the current

Table 3. Effects of metal cations and other chemicals on the activity of GA from *T. thermo-saccharolyticum* ATCC 7956[a]

Reagent	Relative activity (%)[b]			
	0.1 mM	1 mM	5 mM	10 mM
Control (no addition)	100	100	100	100
Ca^{2+}	104	105	107	
Zn^{2+}	59	41	19	
Mg^{2+}	97	103	105	
Mn^{2+}	96	91	88	
Co^{2+}	94	84	65	
Cu^{2+}	92	53	15	
Pb^{2+}	93	61	12	
EDTA		95	98	96
DTT		97	92	88

[a] Pure GA (40 U/mg) was incubated with each substance at the concentrations shown above for 5 min at 60°C.

[b] Relative activity is based on the original activity of GA (100 %) without adding any of the above substances.

methods used in this research seemed the best choice for *T. thermosaccharolyticum* ATCC 7956 GA, which has a low product expression level in the fermentation broth.

However, we still tried to collect a full data set on several crystals of native GA that diffracted to approximately 2.1 Å. Studies of GA heavy atom derivatives showed K_2PtBr_4 derivative is most suitable for further solution of three-dimensional structure. Preliminary analysis of GA crystals suggests that crystals have space group $P2_12_12$ with unit cell parameters of 81.20 Å x 101.97 Å x 164.27 Å. Comparison of these unit cell parameters with those two other GA crystals previously investigated (Aleshin et al., 1994b; Sev [ik et al., 1998) suggests that our crystals contain two molecules of GA in the asymmetric part of the unit cell. Crystallization of GA with noncrystallographic symmetry suggests that it may exist in solution as a dimer under some conditions. More detailed crystallization results can be found in Appendix B.

CONCLUSIONS

The work on GA from the thermophilic strain *T. thermosaccharolyticum* ATCC 7956 described here includes: 1) designing the anaerobic equipment to cultivate this strain; 2) determining the localization of the GA product; 3) scaling up the fermentation from 10 mL Hungate tubes to a 50-L fermentor; 4) GA recovery and purification using two-step chromatography; 5) determining the optimal pH and temperature on the GA activity; 6) studying GA thermostability; 7) investigating the effect of metal cations and other chemicals on the GA activity; 8) measuring the amino acid composition and *N*-terminal sequence; 9) crystallizing the purified GA and trying to solve its three-dimensional structure. The following conclusions can be presented:

- The designed anaerobic equipment and culture conditions worked well for the production of this prokaryotic GA.
- GA from *T. thermosaccharolyticum* ATCC 7956 under these cultivation conditions proved to be mainly an extracellular product.
- The overall two-step purification procedure, acarbose-Sepharose affinity chromatography followed by ion exchange, is superior to other reported methods by requiring fewer purification steps but giving a higher purification (309-fold) and similar recovery (25.8%). The specific activity of the purified GA was 49.4 U/mg.
- The sequence of the first fifteen *N*-terminal amino acid residues is Val-Leu-Ser-Gly-Lys-Ser-Asn-Asn-Val-Ser-Ser-Ile-Lys-Ile-Asp. This sequence is identical to that of GAs from *Clostridium sp. G0005* and *T. thermosaccharolyticum* DSM 571 and 572, but has low similarity with that of other fungal GAs.
- The optimal pH and temperature for GA activity was 5.0 and 65°C, respectively.
- GA showed a very good thermostability at temperatures up to 70°C. However, higher temperatures gave high activity loss.

- Ca^{2+} and Mg^{2+} had no effect on GA activity at 5 mM. Zn^{2+}, Cu^{2+}, Pb^{2+} at 5 mM caused more than 80% inhibition of GA activity. EDTA and DTT hardly inhibited GA activity at 10 mM.
- The best conditions to crystallize this GA are 15% PEG 3350, 100 mM Tris-Cl (pH 8.0), and 200 mM Li_2SO_4 at room temperature.
- Studies of GA heavy atom derivatives showed that the K_2PtBr_4 derivative is most suitable.
- Preliminary analysis of GA crystals suggests that crystals have space group $P2_12_12$ with unit cell parameters of 81.20 Å x 101.97 Å x 164.27 Å. Comparison of these unit cell parameters with those two other previously investigated GA crystals suggests that our crystals contain two molecules of GA in the asymmetric part of the unit cell. Crystallization of GA with noncrystallographic symmetry suggests that it may exist in solution as a dimer under some conditions.

APPENDIX A: GA PRODUCTION FROM *T. thermosaccharolyticum* ATCC 27384

I used another thermophilic strain, *T. thermosaccharolyticum* ATCC 27384 (also called *T. thermosaccharolyticum* DSM 572), to produce bacterial GA before I changed to the current strain. The former strain produced extremely low amounts of GA both in the cells and in the medium, although I expended lots of effort to improve its performance. It showed no promise for further study. All the materials and results related to this strain are listed below.

Strain and Cultivation

T. thermosaccharolyticum ATCC 27384 was obtained from Dr. Reilly's former graduate student, Kendall Cradic, who purchased it from the American Type Culture Collection (Manassas, VA). The same anaerobic methods were used here as before. This spore-forming strain was first "activated" by cultivating it in PRAS-beef liver medium (see below) overnight. It was then transferred to the tubes with PRAS-glycerol medium (0.6% tryptone, 0.2% yeast extract, 0.05% cysteine hydrochloride, 0.0001% resazurin, 50 mL glycerol, 50 mL distilled water, pH 7.0). The tubes were frozen at –70°C for future use. The growth experiment was conducted at 60°C in Hungate tubes and a 2-L fermentor containing 1.7 L of the following medium, which includes (w/v) 1.0% starch, 0.2% yeast extract, 0.6% tryptone, 0.3% KH_2PO_4, 0.4% Na_2HPO_4, 0.04% $MgCl_2 \cdot 6H_2O$, 0.3% NH_4Cl, 0.0001% resazurin, and 0.05% cysteine hydrochloride. The pH was adjusted to 6.7.

Preparation of Beef Liver Medium for Anaerobes

Beef liver (500 g, cut up in small pieces) was put in 1.0 L tap water and soaked overnight in a refrigerator. The fat was skimmed off the top and the remainder was autoclaved for 10 min at 15 psig. It was filtered through cheesecloth and the meat was saved. Then 10 g peptone and 1 g K_2HPO_4 were added to the broth and the pH was adjusted to 8.0. The broth was filtered through filter paper and the volume was adjusted to 1.0 L with more tap water. The

broth was boiled and cooled under 99% oxygen-free argon. Meat was added to a depth of 1/2 inch in tubes already containing 5 mg $CaCO_3$. Broth in 5-mL amounts was dispensed into the meat tubes using anaerobic techniques. The tubes were sealed with butyl rubber stoppers and screw caps and then were autoclaved at 121°C for 25 min.

Growth Curve and GA Localization

Exactly 8% (v/v) of the glycerol-prepared strain of *T. thermosaccharolyticum* was first inoculated into 15-mL Hungate tubes containing 10 mL medium. After 18 h cultivation at 60°C, 4% (v/v) of the above inoculum was added to a 250-mL flask containing 100 mL medium and cultivated for 14 h. Of this growing inoculum, 60 mL was inoculated into a 2-L fermentor containing 1.7 L medium. The metabolic characteristics of this strain were investigated in this small fermentor. The cell density (OD_{580}) and pH of the culture broth were measured every 2 h. After 10 h fermentation, 15-mL samples of the culture broth were taken at several time intervals and centrifuged for 20 min at 5000 x *g* to separate the cells from the supernatant. Activities in the cells and supernatant were measured. The intracellular activity was determined as before.

As shown in Figure 13, *T. thermosaccharolyticum* ATCC 27384 grew well on starch as the only carbon source. It reached its stationary growth phase after 12 h of cultivation. The pH value dropped quickly from 6.4 to 5.2 during the growth phase, but remained almost constant around 5.0 during the stationary phase.

Very low extra- and intracellular GA activities were detected even in the stationary phase (Figure 14) compared to other literature (Specka et al., 1991), suggesting that this particular strain might have intrinsic problems. Its extremely low GA activity prevented its study beyond that described here.

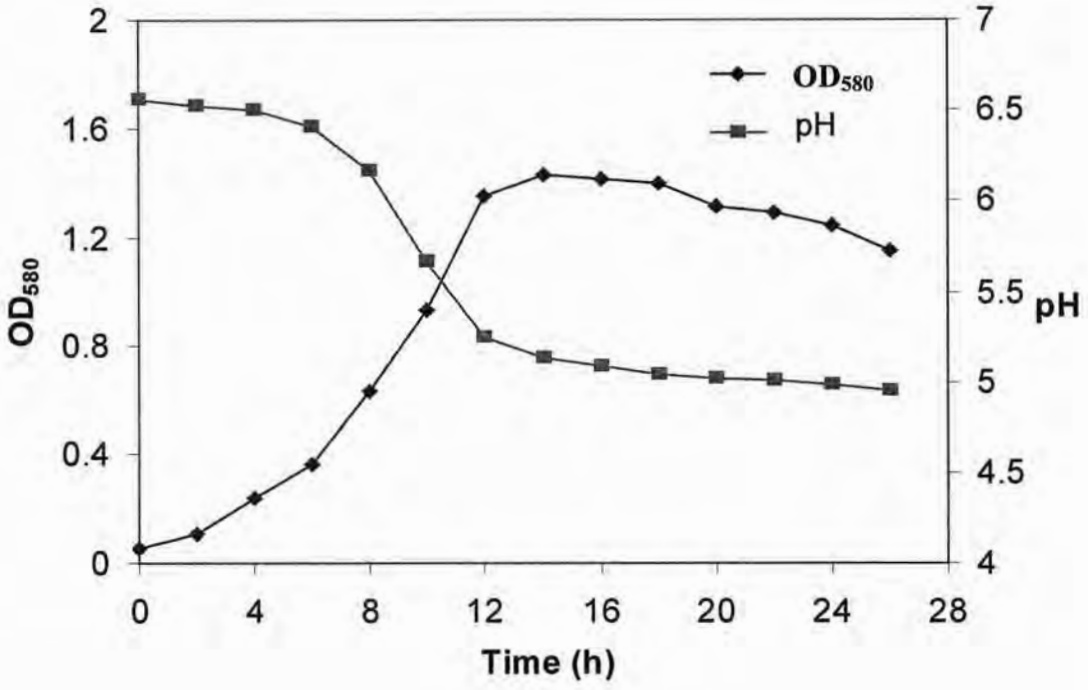

Figure 13. Cell density (OD_{580}) and pH change during growth of *T. thermosaccharolyticum* ATCC 27384 on 1% (w/v) soluble potato starch at 60°C under anaerobic conditions. Growing inoculum (2% (v/v)) was inoculated into 1.7 L medium in a 2-L fermentor. Samples were taken at 2-h intervals for analysis.

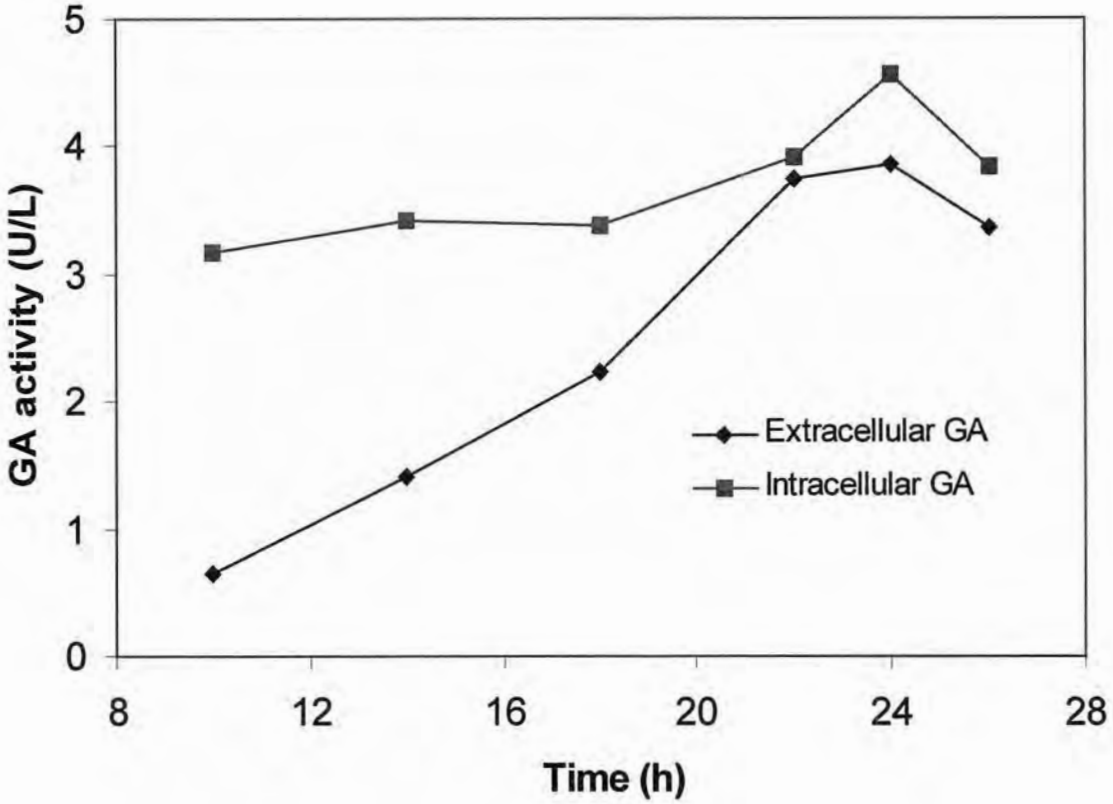

Figure 14. GA production during growth of *T. thermosaccharolyticum* ATCC 27384 on 1% (w/v) soluble potato starch at 60°C under anaerobic conditions. Growing inoculum (2% (v/v)) was inoculated into 1.7 L medium in a 2-L Braun fermentor. Samples of 15 mL culture broth were taken at different time intervals and cells were separated from the supernatant by centrifugation at 5000 x g for 20 min. Cells were disrupted by a French press at 80 Mpa. Both intracellular and extracellular activities were measured by the same enzyme assay.

Effect of Medium pH and Growth Temperature on GA Production

The effect of pHs from 6.4 to 7.4 on GA production was investigated. Cultivation was conducted at 60°C in flasks containing 300 mL of the above medium, which was inoculated by a log-phase inoculum from the Hungate tubes. Although pH values around 6.7 gave maximal GA production after 24 h cultivation, it was still very low (Figure 15).

To investigate the influence of growth temperature on GA production, Hungate tubes containing 10 mL medium at pH 6.7 were cultivated at different temperatures from 45 to 70°C. Growth temperatures from 55 to 65°C gave about the same secretion of GA into the culture both, but growth at 45 and 70°C gave relatively low GA activities (Figure 16).

Dependence of GA Production on Carbon Source

To determine the effect of carbon source and its concentration on the production of GA from *T. thermosaccharolyticum* ATCC 27384, a growth experiment was conducted at 60°C in flask containing 300 mL medium with starch or/and glucose at different concentrations. Figure 17 shows that different starch concentrations did not appreciably affect GA production, but they seemed to influence the activity ratio between intracellular and extracellular GA. Glucose as the carbon source in the medium inhibited GA production, and especially inhibited GA secretion into the culture broth.

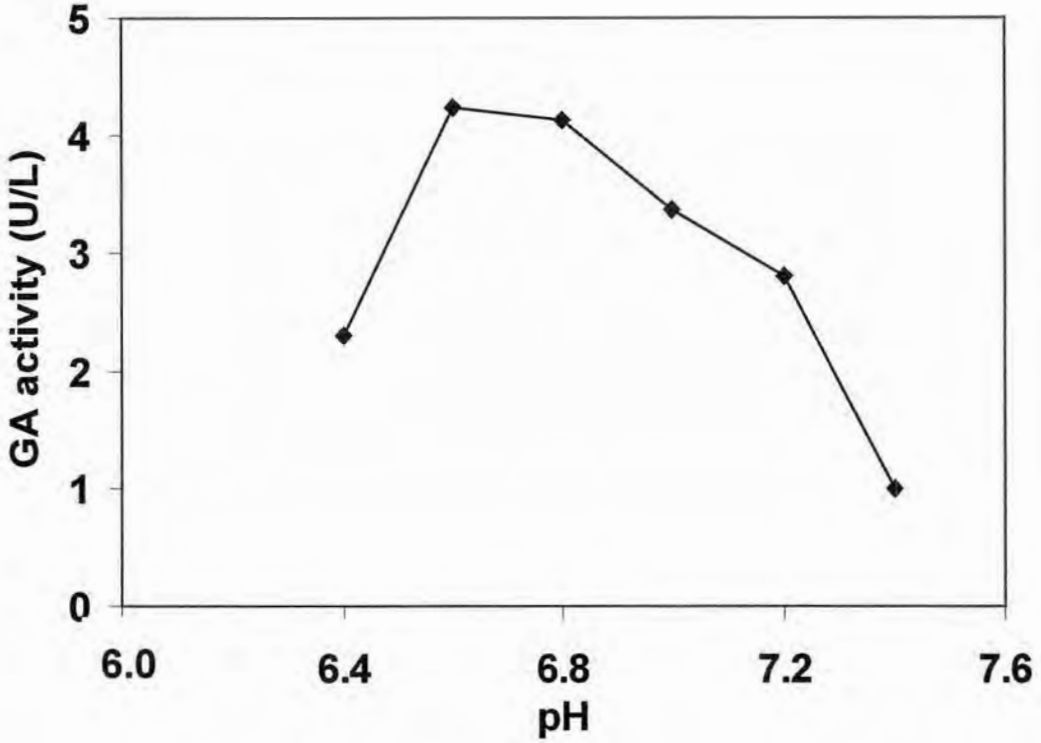

Figure 15. Effect of the original culture medium pH on GA production by *T. thermosaccharolyticum* ATCC 27384. Growth was conducted at 60°C on the same medium containing 1% starch but with different pH values. Of the growing inoculum, 2% (v/v) was inoculated into a flask with 300 mL medium. After 24 h cultivation, cells were separated from the supernatant and intracellular GA activity was measured.

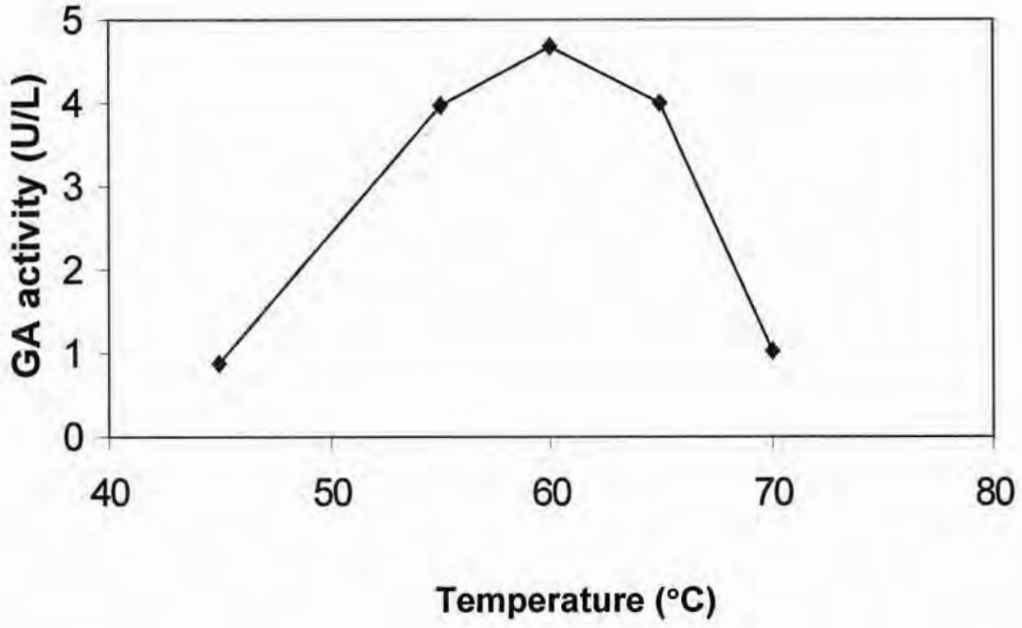

Figure 16. Effect of growth temperature on GA production by *T. thermosaccharolyticum* ATCC 27384. Growth at different temperatures was conducted for 24 h in 15-mL Hungate tubes containing 1% soluble potato starch. Cells were separated from the supernatant by centrifugation at 5000 x *g* for 20 min. Extracellular GA activity was measured.

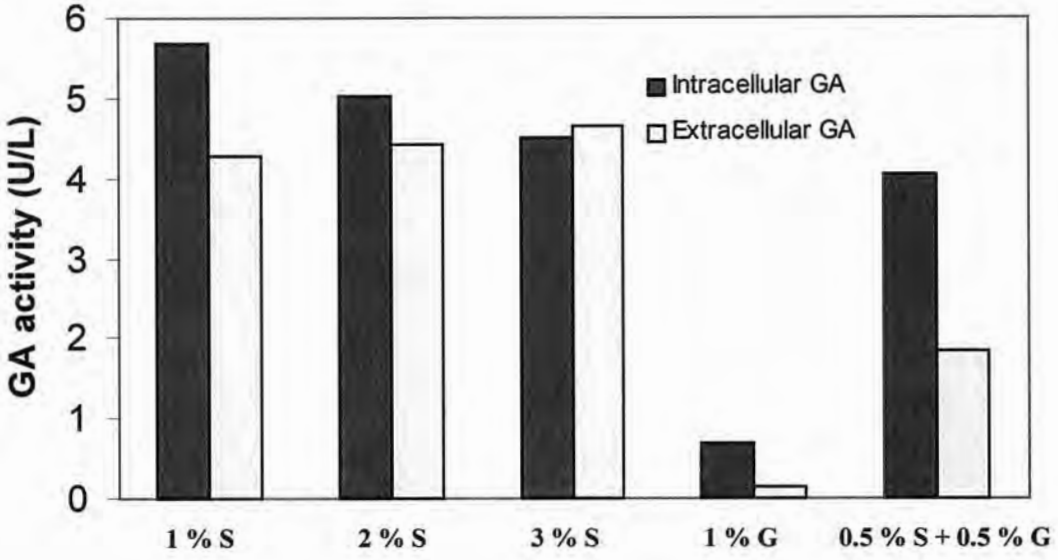

Figure 17. Dependence of GA production by *T. thermosaccharolyticum* ATCC 27384 on different carbon sources (S: starch, G: glucose) and their concentration in the growth medium. The culture was grown at 60°C for 24 h in flasks containing 300 mL medium. After cultivation, cells were separated from the supernatant by centrifugation. Both intracellular and extracellular activities were measured.

APPENDIX B: GA CRYSTALLIZATION (mainly prepared by Alexander Aleshin)

GA Crystallization Conditions and Crystal Treatment

Purified GA, 15–20 mg/mL in 30 mM Tris-HCl buffer, pH 7.5, was crystallized (Materials and Methods). Initial crystallization conditions were found with crystal screen matrices (Jancarik and Kim, 1991; Hampton Research, 2000), and were then optimized by varying precipitant and salt additive concentrations and buffer pH. All trials were done by the vapor diffusion method in hanging drops using Limbro plates (Hampton Research).

The best crystals were produced after filling plate wells with 0.5 mL of the precipitant solution containing 14–16% PEG (polyethylene glycol) 3350, 100 mM Tris-HCl buffer (pH 8.0), and 200 mM Li_2SO_4.

Protein solution (2 μL) was mixed with 2 μL of the precipitant solution on a siliconized glass cover slide. The glass slides were placed on corresponding wells of Limbro plates covered with vacuum grease sealant (Hampton Research). Crystals appeared in 2–4 days and grew to their maximal size during following 1–2 weeks. Crystals shaped as thin plates of 0.2 x 0.2 x 0.02 mm were connected together into large polycrystalline agglomerates.

After crystals reached their maximal size, the cover glasses were opened and 5–20 μL of precipitant solution from the well was added to drops, this being necessary to prevent crystals from drying. Polycrystals were broken into single crystals with the tip of a syringe needle. Individual crystals were transferred into plastic micro-bridges (Hampton Research), where all the following solution exchanges were done.

Preparation of Heavy Atom Derivatives

Background

T. thermosaccharolyticum GA has very little homology with GAs whose structures have been investigated (Ducki et al., 1998). As a result, the solution of its structure by the molec-

ular replacement method is too difficult. We chose the alternative method of isomorphous replacement. The most popular approach today is to grow bacteria on a medium containing selenomethionine and inhibitors of cellular methionine production (Cowie and Cohen, 1957; Hendrickson et al., 1989, 1990). This approach allows a straightforward structure solution by the multiwavelength anomalous dispersion (MAD) method. Unfortunately, the yield of GA from *T. thermosaccharolyticum* is so low that substitution of methionines with selenomethionines is impractical. Therefore, the only available approach for structure solution of our GA was isomorphous replacement with heavy atom reagents.

Analysis of sulfhydryl groups available for modification by DTNB (Materials and Methods) indicated that there is only one such group on the protein surface, with its modification not affecting enzyme activity. This suggests that heavy atom reagents specific for sulfhydryl groups should be most suitable. We tried *p*-chloromercuribenzoic acid (PCMB) to modify the crystals as well as reagents specific for histidine and methionine (potassium tetrabromoplatinate, K_2PtBr_4), aspartate and glutamate (uranyl acetate, UO_2Ac_2), and hydrophobic pockets (Me_3PbAc, trimethyl lead acetate). However, the preliminary data analysis demonstrated that only the K_2PtBr_4 derivative was suitable for further work (Table 4).

Table 4. Concentrations and times used to modify crystals.

Reagent	Concentration (mM)	Soaking time (days)	Comments
PCMB	0.01	2	High crystal mosaicity, not suitable
Me_3PbAc	1	2	No substitution, not suitable
K_2PtBr_4	1	2	Isomorphous substitution, suitable
UO_2Ac_2	5	2	No substitution, not suitable

Freezing Crystals

After chemical modification, crystals were transferred into cryoprotectant solution and flash-frozen in liquid N_2. Because modern synchrotron sources of X-ray quickly damage crystals by producing free radicals in them, crystals must be frozen. However, this would cause the water solution around and inside these crystals to itself crystallize, which is not acceptable. To prevent water from freezing, 25-30% glycerol is usually added as a cryo-protectant.

Thus, native crystals and heavy atom derivatives were transferred to a solution containing 25% glycerol, and after 5 min soaking they were transferred into small loops (0.2 mm) made of 10 μm plastic thread. Loops were mounted on a CrystalCap System (Hampton Research). Crystals in the loops were frozen by submerging them into liquid N_2 and stored in a liquid N_2 dewar until data collection at the National Synchrotron Light Source (NSLS) station in Brookhaven, NY.

Crystals stored in a dry liquid N_2 shipper were delivered to NSLS by Federal Express. Data collection was conducted at the X4C beam line (http://www.nsls.bnl.gov/BeamLine/pages/x4c.html). Most crystals were twins consisting of several disoriented crystals (Figure 18) and thus were unacceptable for data collection. However, we found several crystals of native GA that diffracted to approximately 2.1 Å, and a full data set was collected.

We also analyzed the quality of heavy atom derivative crystals by collecting small data sets (20 frames). These derivative data sets were later compared against native data sets to find suitable derivatives. Tables 5–7 present the quality of the collected data and results of the heavy atom derivative search.

Table 5 presents the deviation of the intensities of the derivative data from the intensities of the native data as a function of resolution (18-3 Å). Tables 6 and 7 presents deviations in the intensities of native and derivative data scaled alone. The large jump in *R*-factors and χ^2

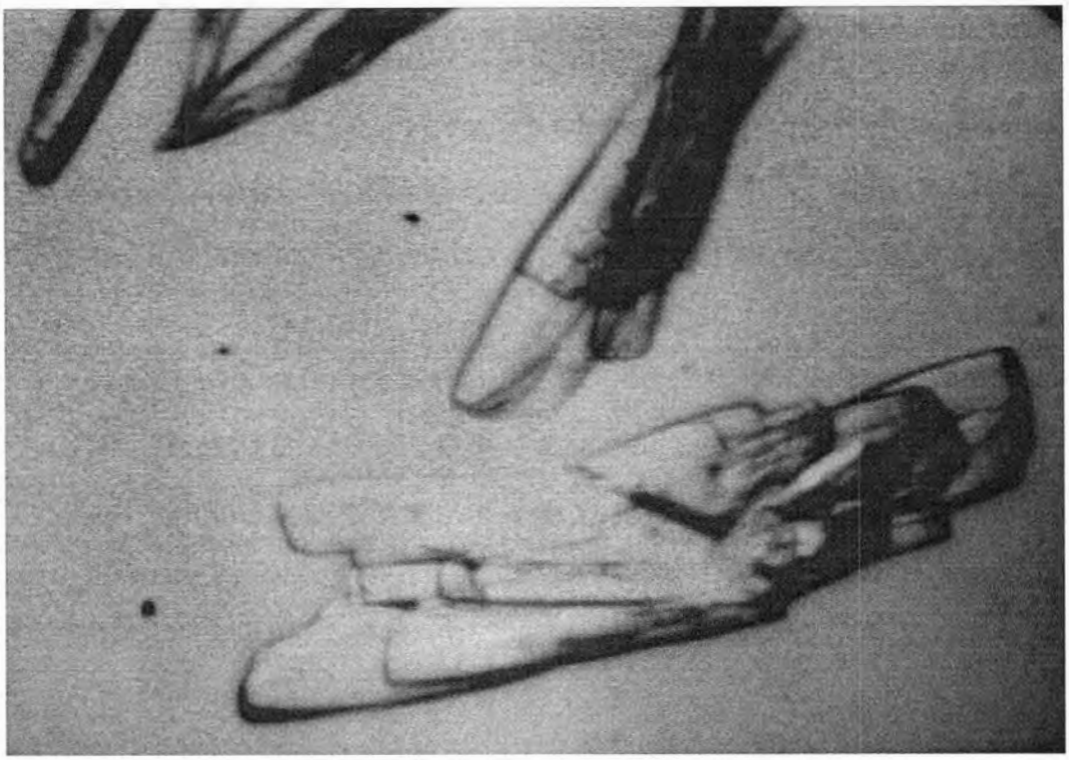

Figure 18. A photograph of *T. thermosaccharolyticum* ATCC 7956 GA crystals. Crystals were produced using the vapor diffusion method in hanging drops by filling Limbro plate wells with 0.5 mL of the precipitant solution containing 14–16% PEG 3350, 100 mM Tris-HCl buffer (pH 8.0), and 200 mM $LiSO_4$.

Table 5. Statistics for substitution of GA crystals by K_2PtBr_4.

Lower limit (Å)	Upper limit (Å)	Average I	Average error	Average statistics	Norm. χ^2	R-factor	R^2-factor
18.00	6.38	3801.1	122.8	117.7	41.507	0.152	0.195
6.38	5.10	2719.0	89.8	85.2	26.005	0.148	0.167
5.10	4.47	4350.3	136.1	129.8	27.566	0.147	0.177
4.47	4.06	4465.8	152.4	145.5	25.724	0.137	0.156
4.06	3.77	3518.1	126.0	121.1	21.945	0.154	0.169
3.77	3.55	3119.7	114.8	110.4	21.740	0.161	0.173
3.55	3.38	2410.9	95.0	93.5	20.035	0.163	0.188
3.38	3.23	1950.3	83.9	83.3	20.384	0.169	0.201
3.23	3.11	1431.0	69.5	69.2	13.118	0.184	0.207
3.11	3.00	1239.3	63.8	63.6	11.291	0.182	0.189
All reflections		2913.3	105.7	102.2	22.898	0.154	0.177

where

I = reflection intensity

$$R-\text{factor} = \frac{\sum |I - \langle I \rangle|}{\sum I}$$

$$R^2-\text{factor} = \frac{\sum (I - \langle I \rangle)^2}{\sum I^2}$$

$$\chi^2 = \frac{\sum (I - \langle I \rangle)^2}{Error^2 \cdot \frac{N}{N-1}}$$

Single measurements are excluded from all sums.

Table 6. Statistics for the native crystal.

Lower limit (Å)	Upper limit (Å)	Average I	Average error	Average statistics	Norm. χ^2	R-factor	R^2-factor
20.00	4.51	3493.7	116.6	66.3	1.965	0.049	0.068
4.51	3.59	3744.3	137.1	76.7	1.286	0.057	0.068
3.59	3.13	2050.0	90.9	62.2	1.138	0.073	0.076
3.13	2.85	1105.5	64.8	55.1	1.081	0.098	0.096
2.85	2.64	681.6	58.7	54.0	1.018	0.134	0.227
2.64	2.49	524.1	58.7	55.6	0.867	0.163	0.166
2.49	2.36	395.9	59.7	57.8	0.837	0.207	0.236
2.36	2.26	347.8	61.9	60.3	0.802	0.239	0.558
2.26	2.17	309.5	68.4	67.0	0.774	0.269	0.281
2.17	2.10	265.8	75.9	75.0	0.973	0.322	0.000
All reflections		1326.2	79.7	62.9	1.093	0.087	0.087

Table 7. Statistics for the K_2PtBr_4 derivative.

Lower limit (Å)	Upper limit (Å)	Average I	Average error	Average statistics	Norm. χ^2	R-factor	R^2-factor
20.00	5.35	10084.4	513.6	364.7	0.909	0.035	0.038
5.35	4.26	11038.2	550.5	367.9	1.659	0.053	0.055
4.26	3.73	9242.1	499.2	350.2	2.148	0.066	0.069
3.73	3.39	6630.0	405.9	304.3	2.130	0.079	0.080
3.39	3.15	4051.7	323.1	267.9	1.678	0.092	0.088
3.15	2.96	2649.1	280.7	247.8	1.143	0.107	0.097
2.96	2.81	1873.1	245.7	237.6	1.151	0.136	0.122
2.81	2.69	1321.5	234.9	232.8	0.986	0.168	0.144
2.69	2.59	1092.5	239.3	237.7	0.951	0.209	0.215
All reflections		5393.3	368.0	291.1	1.308	0.065	0.061

indicates that K_2PtB_4 is bound to the crystal. The decrease of χ^2 with resolution indicates that the substitution is isomorphous.

Preliminary Results

The K_2PtBr_4 derivative is most suitable. This finding will allow us to solve the GA structure after a full data set for this derivative crystal is collected. Data collection is scheduled on March 17 at the IMCA 17ID beam line of Argonne National Laboratory.

Preliminary analysis of GA crystals suggests that crystals have space group $P2_12_12$ with unit cell parameters of 81.20 Å x 101.97 Å x 164.27 Å. Comparison of these unit cell parameters with those two other GA crystals previously investigated (Aleshin et al., 1994; Sev [ik et al., 1998) suggests that our crystals contain two molecules of GA in the asymmetric part of the unit cell. Crystallization of GA with noncrystallographic symmetry suggests that it may exist in solution as a dimer under some conditions. Noncrystallographic symmetry will simplify the structure solution for *T. thermosaccharolyticum* GA.

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